BIOMARKERS OF AGEING AND FRAILTY IN THE DIALYSIS COHORT

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MBBS MRCP

Submitted in partial fulfilment of the requirements of the Degree of Doctor of Philosophy

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Statement of originality

I, **Vasantha Muthu Muthuppalaniappan**, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below, and my contribution indicated. Previously published material is also acknowledged below.

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Abstract

INTRODUCTION: There appears to be an accelerated ageing process seen among patients with end stage kidney disease. They often exhibit premature aged phenotypes which include frailty, sarcopenia and protein energy wasting. These phenotypes are associated with increased morbidity and mortality. The exact reason for premature ageing in this cohort has been poorly understood. We hypothesised that biomarkers of cellular senescence and biological age may be associated with features of ageing observed in dialysis patients; in particular the frailty phenotype.

OBJECTIVES: The aim of the study was to investigate the relationship between biomarkers of ageing; telomere length (TL) and DNA methylation (DNAm) status with frailty phenotype.

METHODS: All patients had their DNA extracted from peripheral leucocytes and frailty was measured by using the Fried Frailty Phenotype criteria. Extracted DNA was used to measure TL by quantitate polymerase chain reaction and DNAm status was measured by sodium bisulphite conversion with targeted sequencing of 48 CpG sites. Dialysis patients were followed up after a year for repeat telomere length and frailty assessment.

RESULTS: Between the period of December 2015 to July 2018, 339 patients were recruited. Of these 335 patients had TL and 253 patients had DNAm status measured successfully. Frailty assessment at baseline were completed in 299 patients and 155 patients at 1 year. Repeat TL at 1 year was measured in 136 patients. A univariate

analysis found that baseline TL was longer in the control group of healthy donors in comparison to dialysis patients, p=0.006 but this significance was lost after adjusting for age and gender. A decrease in mean TL (p=0.001) and increased mean DNAm age (p=0.001) was observed in the frail group. TL and DNAm age were significantly associated with frailty in a univariate analysis, p=0.010 and p= 0.014 respectively but only TL remained significant in a multivariate analysis to predict frailty, p=0.018. A receiver operating characteristic curve analysis demonstrated that TL was a significant predictor of frailty, p=0.0010 with an area under the curve of 0.64. A decrease of TL by 1 standard deviation was associated with a 52.2% increase risk of frailty when adjusted for age and gender in the dialysis cohort.

CONCLUSION: The study supports the hypothesis that TL; a biomarker of ageing is better associated with frailty, an ageing phenotype in comparison to DNAm age in dialysis patients.

(Word count: 297 words only)

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List of Abbreviations

AMAR	DNA methylation age/chronological age
AUC	Area under curve
BMI	Body mass index
Bp	Base pair
BS	Bisulphite conversion
CCI	Charlson's Comorbidity Index
CKD	Chronic Kidney Disease
CRP	C-reactive protein
CV	Coefficient of variation
D-loop	Displacement loop
Delta T/S	Telomere attrition
DNA	Deoxy ribonucleic acid
DNAm	DNA methylation
eGFR	Estimated glomerular filtration rate
ESKD	End stage kidney disease

FISH	Fluorescence in situ hybridisation
G4’s	Guanine quadruplexes
GH	Growth Hormone
Hb	Haemoglobin
HD	Haemodialysis
IFC	Fluidigm Access Array
IGF	Insulin growth factor
IL-6	Interleukin 6
Kbp	Kilobase pair
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PD	Peritoneal dialysis
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RRT	Renal replacement therapy

RT-qPCR	Real time qPCR
sCr	Serum creatinine
SD	Standard deviation
SCG	Single copy gene
SE	Standard error
T-loop	Telomere loop
T/S ratio	Telomere length measured by qPCR
TER	Telomerase RNA
TERT	Telomerase reverse transcriptase
TL	Telomere length
TRF	Terminal restriction fragments
TRF 1	Telomeric repeat factor 1
TRF 2	Telomeric repeat factor 2
WCC	White cell count
ZT/S	Standardised T/S ratio

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CHAPTER 1: GENERAL INTRODUCTION

1.1 The Biology of Ageing

1.1.1 What is ageing?

Ageing is a process of growing old. However, published literature has shown that the definition of ageing is much more complex and arguably the least well understood aspect of human biology. Ageing itself is a complicated process with multiple hypotheses with more than 300 'theories' of ageing described in the past (Medvedev, 1990). Fortunately, recent advances in ageing research have simplified some of the theories. Despite this, and due to the intrinsic complexity of the ageing process there remains uncertainty about many factors and processes involved in its aetiology.

Ageing appears to be a time dependant process characterized by progressive decline and gradual deterioration of physiological functioning across multiple organ system necessary for survival and fertility (Kirkwood, 2005). It also represents a normal decline in physical, social and cognitive function with age, heightened by extrinsic factors. The current challenge in the field of ageing is the lack of standardised parameters available to measure ageing and allow its quantification.

1.1.2 Biological age versus chronological age

All cultures have anecdotes about the relationship between one's chronological age and biological age. "Age is just a number" and "You are as old as you feel" are some of the quotes that highlight the difference between biological and chronological age. The outcome from The Dunedin Multidisciplinary Health and Development Study (Belsky et al., 2015) have reinforced existing findings that support the notion that every human being has a chronological age and biological age.

Chronological age is the record of time elapsed since birth which represents the period of time an organism exists. Biological age also referred to as physiological age or functional age is a concept used to reflect ageing that occurs at a cellular level. Biological age occurs at varying rates within individual species as it is affected by various factors intrinsic and extrinsic factors e.g. lifestyle, genetics, exercise, sleep habits etc. (See Figure 1: Biological age vs chronological age). Biological age is a superior index for disease related mortality than chronological age. This is because biological age takes into account factors that can affect a physical state of an individual's health status which chronological age does not.

Levine and Crimmins showed that biological age could predict 10-year mortality more accurately than other measures, such as Allostatic Load and Framingham Risk Score using 9942 subjects (Levine and Crimmins, 2014).

Allostatic load is defined as the cost of chronic exposure to fluctuating or heightened neural or neuroendocrine response resulting from repeated or chronic environmental challenge that an individual reacts to as being stressful (McEwen and Stellar, 1993). A study among 557,940 Koreans between 20-93 years revealed that, the average biological age was almost the same as the average chronological age in those that survived during the study duration (Yoo et al., 2017). However, in those that died during the 17 year follow up period, the biological age was higher than the chronological age with the death rate significantly higher as gap between the biological and chronological age widened (Yoo et al., 2017). Therefore, biological age may be a better proxy for the ageing phenotype.

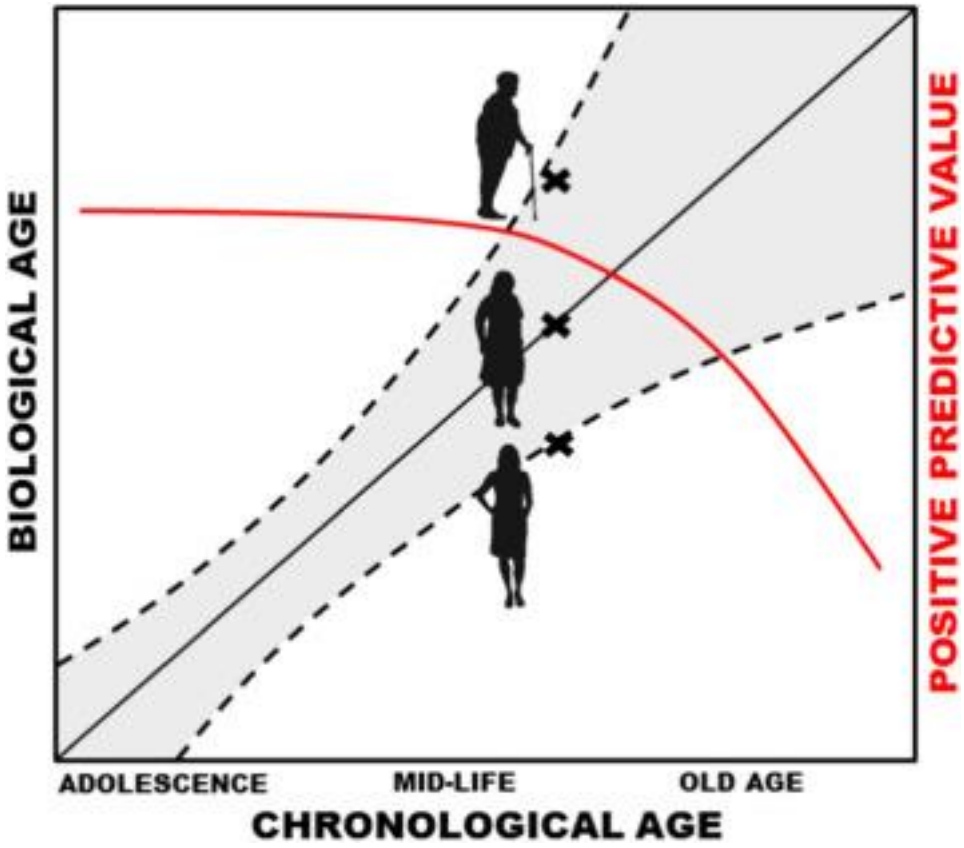

Figure 1: The concept of biological age predictors

A biological age predictor could be defined as a biomarker correlated with chronological age (black line), which brings additional information in the risk assessments for age-related conditions on top of chronological age. Hence, adult individuals of the same chronological age could possess different risks for age-associated diseases as judged from their biological ages (x's in figure). Usually, the positive predictive value (red line) of a biological age predictor decreases from mid-life and onwards due to the increased biological heterogeneity at old age (confidence interval described by dashed lines increases at old age) (Jylhävä, Pedersen and Hägg, 2017). Permission not required as for non-commercial use. Refer to Creative Commons Attribution-Non Commercial-No Derivatives License (CC BY NC ND).

Biological age has been proposed as a proxy for ageing. There is currently no standardised or general consensus on biomarkers accepted for estimating biological age. However, the American Federation for Ageing Research have proposed a clear set of requirements for a biomarker of ageing as outlined below (Johnson, 2006)(Sharman and Zhumadilov, 2011):

1) It must predict the rate of ageing and provide information about the functional condition of the body, its metabolic and regulatory systems as well as a better predictor of lifespan than chronological age.

2) have quantitative characteristics that underlies the ageing process, hence correlates with age and not the effect of disease

3) be reproducible, sensitive, and specific without causing any harm to a person

4) be suitable for use in humans as well as in laboratory animals so it can be tested in animal models prior to being validated in humans

There are currently 6 potential types of recognised biological age predictors and are referred to as 1) epigenetic clock 2) telomere length 3) transcriptomic predictors 4) proteomic predictors 5) metabolomics-based predictors 6) composite biomarker predictors.

The biomarkers of interest explored in this thesis are the epigenetic clock better known as DNA methylation (DNAm) and telomere length (TL) as both were the most plausible candidates out of all the existing predictors of biological age. TL and DNAm techniques have been well developed, cost effective and practical for the use of large-scale studies in comparison to the other predictors of biological age. Both of these biomarkers have been tested in different tissues and validated in independent cohorts with more than a 1000 studies on TL and more than a 100 studies on DNAm (Jylhävä, Pedersen and Hägg, 2017). TL is extensively validated but has a low predictive power of mortality whilst DNAm is thought to be a stronger predictor but not validated enough. Other biological age predictors are useful but have not been well studied as TL and DNAm and still requires further independent validation. TL and DNAm has been shown to provide additional evidence of individual ageing independent of chronological age as they successfully predict mortality, morbidity and physical function which is the basis of this PhD project (Refer to Chapter 2: Aims). These are the reasons for favouring TL and DNAm over all the other available biomarkers of ageing.

1.1.3 Cellular senescence and replicative senescence

The ageing process at a cellular level is known as cellular senescence which is a halt in permanent growth and leads to cellular apoptosis while the lifespan of a cell is often defined by replicative senescence which is the decline in the fitness of the mother cells for further proliferation (Currais, 2017). Senescence was described by Hayflick and Moorhead in 1961 when they discovered that primary human cells would only be able to divide a finite number of times before dying (Hayflick and Moorhead, 1961). The term given to this phenomenon describing cell specific limited capacity of replications, is the 'Hayflick Limit'. This is only reported in normal mammalian cells unlike immortal, abnormal or cancer cell lines. Once the limited capacity for replication has been reached, cells than undergo replicative senescence.

Hayflick and Moorhead suggested that the phenomena of limited replicative capacity could represent how old a cell is, based on how many times a cell underwent division and this could be linked to ageing (Hayflick and Moorhead, 1961)(Hayflick, 1965) (Hayflick, 1979). The mechanism underlying the phenomenon of Hayflick limit was subsequently described as DNA end replication problem (See Figure 2).

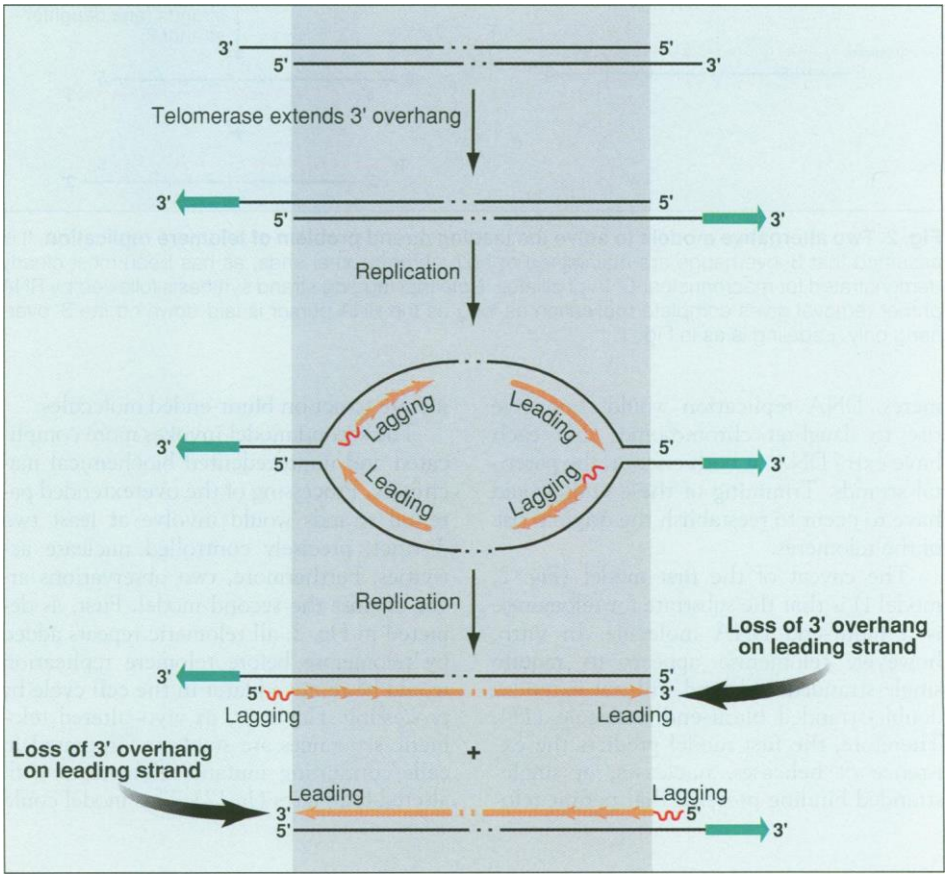

Figure 2: The leading strand problem of telomere replication also known as the end replication problem

Overextension of parental telomeres by telomerase followed by semi conservative deoxyribonucleic acid (DNA) replication does not regenerate a 3’ overhang on the leading strand. Sequence added by telomerase is shown by the thick arrow in green. Ribonucleic acid (RNA) primers are indicated by wavy line in red. Parental DNA is indicated by thin lines in black and replicated by the DNA indicated by the thicker lines in orange. Arrows in black indicate the polarity of DNA 5’ to 3’.Reproduced with permission (Lingner, Cooper and Cech, 1995).

1.2 Telomere Biology

1.2.1 A history of Telomeres

Seminal observations by Muller and McClintock revealed that broken chromosomes were unstable and prone to rearrangements and fusion (Muller, 1938) (McClintock, 1939). Protecting chromosome ends from such events highlighted the importance of the terminal end of the chromosome as key to cell survival and growth (Olovnikov, 1973) (Olovnikov, 1996). However, the molecular nature of these structures was yet to be discovered. The discovery of the structure of DNA by Watson & Crick in 1953 was a key first step that lead to a greater understanding of the molecular nature of terminal section of the chromosome (Watson and Crick, 1969). The end of the chromosome was named telomeres from the origin of the Greek word ‘telo’ meaning end and ‘meros’ meaning part. It was proposed that the finite doubling capacity of normal mammalian cells is due to a loss of telomeric DNA and eventual deletion of essential sequences (Olovnikov, 1973) (Lundblad and Szostak, 1989).

1.2.2 The end replication problem

The underlying mechanisms that controls this phenomenon were described by Calvin Harley in 1990 (Harley, Futcher and Greider, 1990) who implicated the loss of telomeric DNA as a potential mechanism of senescence. Harley demonstrated that the amount and length of telomeric DNA in human fibroblasts negatively correlated with serial passages during ageing in vitro and possibly in vivo (Harley, Futcher and Greider, 1990). This mechanism was described as the end replication problem that was proposed by Olovnikov and Watson back in 1970s. (Olovnikov, 1973) (Watson and Crick, 1969). The end replication problem refers to a hitch that arises when DNA polymerase, the enzyme responsible for DNA replication is unable to fully replicate the 3' end of the parental DNA strand due to the absence of a template strand. (See Figure 2). This results in telomere shortening with each round of replication as DNA is replicated during each cell division. This mechanism would explain replicative senescence (Olovnikov, 1973). Consequently, each replicative cycle of the cell results in the erosion of the parental guanine-rich 3' end and telomeric DNA progressively shortens in the absence of any compensating mechanism. The shortening by approximately 50-100 bases per round of cell division continues until the critical state of replicative senescence is reached, when no further replication can take place.

1.2.3 The structure and function of telomeres

The telomere is a complex nucleoprotein located at terminal end of each linear eukaryotic chromosome (See Figure 3 and Figure 4).

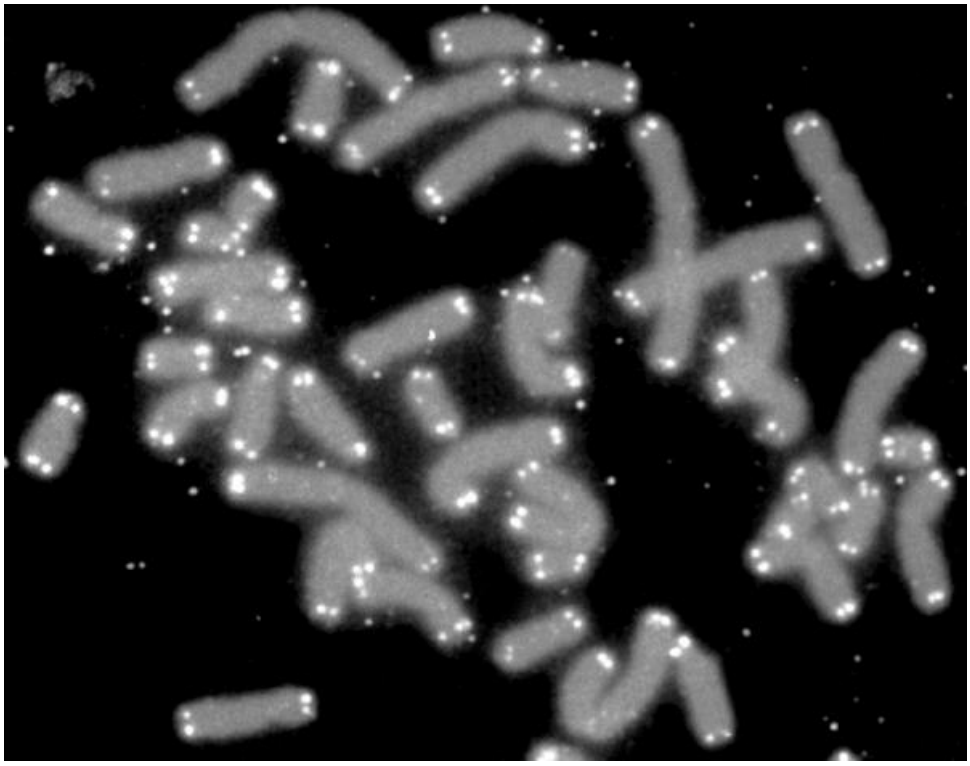

Figure 3: Telomeres appear as the bright spots at the ends of each chromosome in the picture shown above. Image credit by U.S. Department of Energy Human Genome Program (public domain)

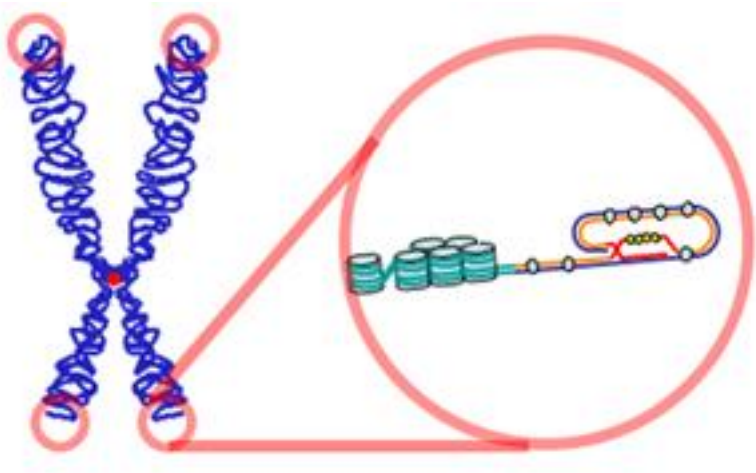

Figure 4: Telomere caps at the end of chromosomes. Image credit by Wikipedia

Due to telomere's stability, unlike the rest of the chromatin, it is not subjected to degradation, recombination or fusion with other chromosomal ends. Telomeres are tandem repeats of guanine enriched strands with a repetitive sequence of TTAGGG in humans. The guanine rich strand of telomeric DNA is always in the direction of 5'-3' towards the terminal portion of the chromosome and contains approximately 200 nucleotides as a consequence of end terminal replication. Telomere caps protect the end of mammalian chromosomes to prevent the ends from being identified as double strand breaks in DNA, thus limiting chromosome shortening and recombination. Telomere itself does not carry any genetic information but preserves genetic integrity of the chromosome by prevention of degradation and end to end fusion.

In humans, the telomere terminus consists of 4 to 15 kilo base pair (kbp) of the hexanucleotides 5′-TTAGGG-3′ (Von Zglinicki, 2002)(Houben et al., 2008). Telomeres undergo attrition with each somatic cell division by approximately

20-200 base pairs (bp), hence their length is an indicator of replicative history and replicative potential of these cells; serving as a mitotic 'clock' (Harley, 1991) (Houben et al., 2008). As little as 400bp of TTAGGG repeats can lead to the formation of a fully functioning telomere (Palm and de Lange, 2008). Experiments demonstrate a strong association between telomere shortening and a reduction of the replicative life span of human cells invitro, consistent with the early genetic evidence obtained in model systems that short telomeres induce senescence (Blackburn, Greider and Szostak, 2009). There are a number of possible mechanisms for loss of telomeric DNA during ageing, including incomplete replication, degradation of termini (specific and non-specific) and unequal recombination, coupled to selection of cells with shorter telomeres (Harley, Futcher and Greider, 1990).

1.2.3.1 Structure of telomeres: Guanine Quadruplexes

The extreme 3' ends (of the 5' to 3' guanine-rich strand) of all telomeres are single stranded, with most studies concurring that the average length of human telomere is 100-200 bases. (Wright et al., 1997) The 3' protruding G rich strand can form complex structures known as guanine-quadruplexes. The G-quadruplexes assume different conformation (Spiegel, Adhikari and Balasubramanian, 2019). (See Figure 5) The multi-stranded structures are held together by square planes of four guanines (G-quartets) interacting by forming Hoogsteen hydrogen bond.

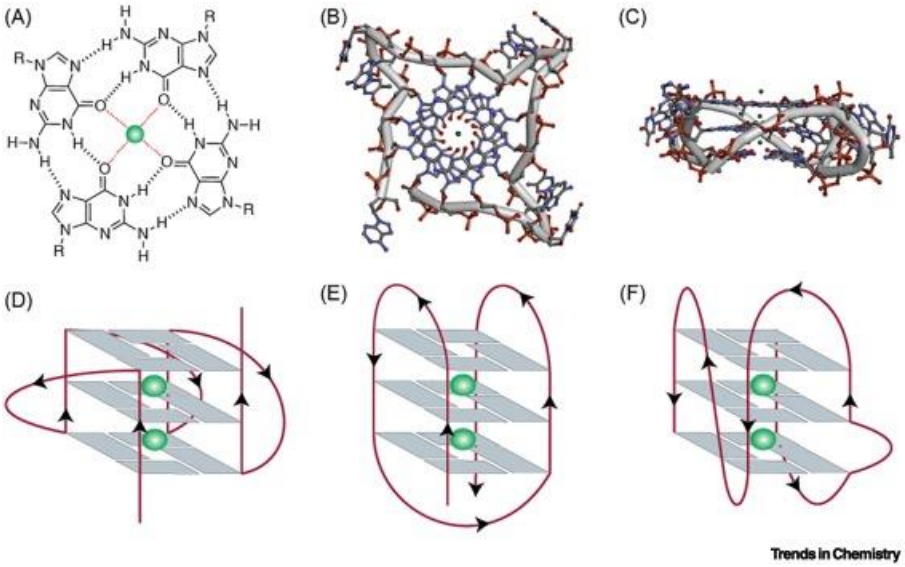

Figure 5: Structure of guanine-quadruplexes

(A) Structure of a G-quartet formed by the Hoogsteen hydrogen-bonded guanines and central cation (coloured green) coordinated to oxygen atoms. Crystal structure of human telomeric guanine quadruplexes G4s (B) top view (C) side view with backbone is represented by grey tube and the structure are colour-coded by atoms. Schematic representation of unimolecular G4s based on the strand direction: (D) parallel, (E) anti-parallel and (F) hybrid with a bulge. (Spiegel, Adhikari and Balasubramanian, 2019) Permission is not required for this non-commercial use. Creative Commons Attribution-Non-Commercial-No Derivatives License (CC BY NC ND).

1.2.3.2 Structure of telomeres: Telomere (T) loop and Displacement (D) loop

Telomeres are not linear and can form looped fold back structures whose stability depends, in a large part, on the involvement of the telomeric repeat factor 1 (TRF1) and telomeric repeat factor 2 (TRF2) proteins (Griffith et al., 1999). Telomeres form large loop structures called telomere loops, or T-loops which sequesters the chromosome terminus. Existence of T-loops provide an explanation of how single stranded chromosome ends are protected from degradation, recombination and fusion (Palm and de Lange, 2008). At the very end of the T-loop the single stranded telomere DNA is held onto a region of double stranded DNA by the telomere strand disrupting the double helical DNA and base pairing to one of the 2 strands call a displacement loop or D-loop, thus protecting the chromosome terminus (See Figure 6).

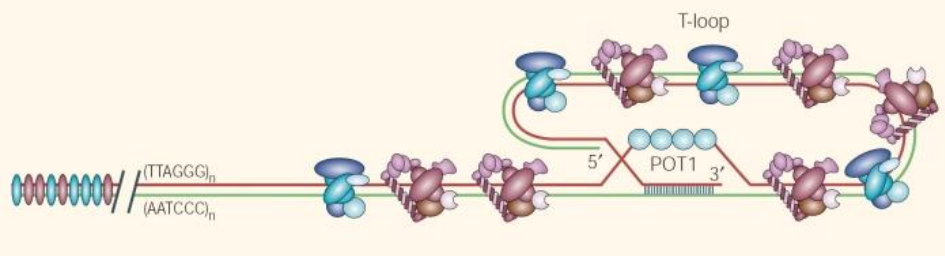

Figure 6: The proposed structure of human telomeric end.

Human telomeres are comprised of a 230kb array of duplex TTAGGG repeats, ending in a 100 to 200 nucleotide protrusion of a single stranded TTAGGG repeats. This DNA can exist as a t-loop in which the overhang invades the duplex-repeat array forming a displacement (D) loop of TTAGGG repeats. Other configurations cannot be excluded.

POT1 binds to the single-stranded TTAGGG- repeat DNA. Two double stranded TTAGGG repeat binding factors (TRF's), TRF1 and TRF2 are associated with the duplex repeats. Reproduced with permission (De Lange, 2004).

1.2.4 Telomerase the historical perspective

Telomere length (TL) is regulated by the enzyme telomerase that maintains telomere stability and telomere shortening. Blackburn, Szostak and Greider were awarded the Nobel Prize in Physiology and Medicine in 2009 for their discovery on how chromosomes are protected by telomeres and the discovery of the enzyme telomerase (Blackburn, Greider and Szostak, 2009). Telomerase enzyme is essential for genomic stability and is the mechanism by which the ends of chromosomes are maintained and spared from erosion or rearrangement during repeated cellular divisions.

1.2.4.1 The structure and function of telomerase

Telomerase is a unique cellular reverse transcriptase that contains an integral ribonucleic acid subunit (telomerase RNA, also known as TER) and a catalytic protein subunit telomerase reverse transcriptase (TERT) as well as several species-specific accessory proteins. The function of telomerase is to extend the 3' of the linear chromosome by synthesizing multiple copies of the specific DNA telomere repeat sequence using a complementary template. The telomerase reaction cycle consists of 3 basic steps (Wyatt, West and Beattie,

2010). The first step is primer recognition followed by binding. Once the primer has bound, the telomeric repeats are then synthesized. This is then followed by translocation and realignment of the new DNA 3' to initiate the next round of telomere synthesis.

Data from knockout mice has shown that lack of telomerase activity causes telomeres to shorten more rapidly over time leading cell cycle arrest and senescence (Lee et al., 1998)(Blasco et al., 1997). Telomerase activity is pathologically high in cancer cells, moderate in germ cell and stem cell line but mostly undetectable in somatic cells (Armanios and Blackburn, 2012). TER subunits contain 2 conserved structural elements; the catalytically essential pseudoknot-template core domain and a stem-loop element called CR4-CR5 (Sandin and Rhodes, 2014). These 2 conserved TER structural elements have been shown to interact directly with TERT (Mitchell and Collins, 2000) (Gardano et al., 2012).

1.2.4.2 Shelterin Complex

Telomeres are bound by a six-protein complex called shelterin complex; comprised of TRF1 and TRF2 which in turn recruits RAP2, TIN2, TPP1 and POT1 (Protection of telomeres 1) that have been implicated in the formation and maintenance of telomere structure by preventing DNA damage and regulating telomerase activity (Palm and de Lange, 2008). TRF1 and TRF 2 are negative regulators of telomere length (TL). Both these proteins inhibit

telomerase activity to inhibit telomere elongation. TRF1 and TRF2 are both involved in the ataxia telangiectasia mutated (ATM) and ATM Rad3-related (ATR) pathway. These pathways are sensors of DNA damage and induce cell cycle arrest upon DNA damage.

1.2.5 Mechanisms of telomere shortening and repair/Regulation of telomeres

The mechanisms regulating TL by telomerase are poorly studied despite much work having been done regarding the complex telomere/telomerase structure.

1.2.6 Telomeres in research and factors affecting it

Telomeres have 3 main features that highlights the reasons for its popularity in the field of research; a) telomeres shorten with each cell division b) telomeres are shortened by oxidative damage c) telomeres reach a critical length and induces a senescent state in cells. These properties of telomeres have formed the focus and foundation of research investigating mechanisms of ageing, age-related diseases and mortality. Both basic science research and clinical studies have demonstrated various factors that are associated with TL not limited to genetic inheritance, environmental and lifestyle factors. Factors that have been consistently shown to affect TL are described below bearing in mind that some factors identified have shown conflicting and inconsistent findings.

1.2.6.1 Age

Both animal and human studies have shown that TL declines with age. A systematic review of TL and age among 124 cross-sectional studies and 5 longitudinal studies revealed a statistically significant inverse correlation between mean age and mean TL for both absolute ($r = -0.338$, $p < 0.0001$) and relative TL ($r = -0.295$, $p < 0.01$) (Müezzinler, Zaineddin and Brenner, 2013). Weighted linear regression from this meta-analysis yielded an estimated telomere loss rate of 24.7 BP/year ($p = 0.0071$) and 0.010 T/S ratio/year ($p = 0.0071$). (Müezzinler, Zaineddin and Brenner, 2013) This rate may not be constant throughout life, although it has been a common practice to describe TL as a linear function of donor age (Frenck, Blackburn and Shannon, 1998). Inverse relationship between age and TL is a consistent observation seen in the literature. However current evidence is insufficient to determine or provide an exact quantification of a reliable TL range for different age groups in healthy people.

1.2.6.2 Gender

There have been inconsistencies in the association between gender and TL in the literature as data on variation according to gender remains sparse. The hypothesis that women have longer telomeres have risen from strong biological evidence supporting the hypothesis that women have longer TL in comparison to men (Aviv et al., 2005). This arises from the role of oestrogen

which has been shown to stimulate telomerase activity (Kyo et al., 1999) (Barrett and Richardson, 2011) via human TERT (hTERT) gene expression, while androgens appear to be a negative regulator of telomerase in normal prostate tissue (Bayne and Liu, 2005). Oestrogen also has a protective effect against reactive oxygen species (ROS) damage (Aviv, 2002) as oestrogen is thought to possess anti-oxidant properties that protects telomeres from extensive oxidative stress induced damage (Von Zglinicki, 2002).

A qualitative meta-analysis exploring the role of TL and gender found that males did have shorter telomeres than females (Barrett and Richardson, 2011). This was also supported by Muezzinler et.al. showing that females of similar ages had longer TL than men, again findings which have not been seen consistently throughout all studies (Müezzinler, Zaineddin and Brenner, 2013). A systematic review and meta-analysis of 36230 participants showed that gender was associated with TL with females having longer TL than males on average (Gardener and et al., 2014). However there was significant heterogeneity between the studies detected and the authors found that different measurement methods affected TL difference in gender. This systematic review revealed that the fluorescence in situ hybridisation (FISH) and polymerase chain reaction (PCR) based methods showed no difference between gender (Gardener and et al., 2014).

It has been suggested that longer TL in females might be due to a slower rate of telomere attrition in females (Okuda et al., 2002) as observed in a cross sectional study (Bekaert et al., 2007) and a longitudinal study (Chen et al.,

2011). However, there have also been studies to dispute these findings with no significant gender-related differences in the rate of age-dependent telomere attrition (Benetos et al., 2001) (Cawthon et al., 2003). One meta-analysis found that the evidence for sex-specific correlation coefficients and yearly telomere loss rates showed no statistically significant differences and the effect of gender on TL was not consistently observed in all studies (Müezzinler, Zaineddin and Brenner, 2013).

Therefore, this may suggests that perhaps female new-borns may have longer telomeres than male at birth which has been observed in one study using flow fish (Aubert et al., 2012) but not seen in another study that used terminal restriction fragments (TRF) southern blotting (Okuda et al., 2002). There are limited studies available in the first 2 decades of an individual which may be crucial in answering the dilemma of TL and gender.

1.2.6.3 Oxidative stress and inflammation

Oxidative stress has been reported to accelerate telomere shortening occurring during DNA replication, ultimately promoting senescence. Oxidative DNA damage constitutes the majority of DNA damage in human cells, causing base damage and unrepaired nucleotides. The oxidative damage to nucleotides and bases accumulate over the life span of a cell or an organism and contributes to senescence (Houben et al., 2008). Senescent cells contain 30% more oxidatively modified guanines in their DNA and 4 times as many free 8-oxodG

bases; a biomarker of oxidative stress (Chen et al., 1995). A study comparing young and senescent cultured human fibroblasts revealed an increase in ROS levels, dysfunctional mitochondria, more DNA double stranded breaks and shorter telomeres in the senescent cells (Passos et al., 2007).

Telomeres are particularly vulnerable to oxidative damage. Petersen et.al. found that repair in telomeres was much slower and incomplete than the rest of the genome when human fibroblasts were exposed to oxidative stress causing an increase in the frequency of single stranded breaks and telomere shortening (Petersen, Saretzki and Von Zglinicki, 1998). In studies by Okaiwa et.al., exposure of DNA to 8-oxodG, (a biomarker of oxidative stress) not only showed that 8-oxodG accumulated with age but an increased level was found in DNA fragments containing telomere sequences (Oikawa and Kawanishi, 1999)(Oikawa, Tada-Oikawa and Kawanishi, 2001). Further evidence for the role of oxidative stress accelerating telomere shortening was observed in human fibroblast when the onset of cellular senescence was correlated with cellular oxygen exposure (Ahmed and Lingner, 2018).

Oxidative damage at random positions within telomeres interferes with the replication fork resulting in stochastic and irregular telomere shortening events (Von Zglinicki, 2002) (Von Zglinicki, Pilger and Sitte, 2000). Oxidative damage also affects telomeres by enhancing the end replication problem which then interferes with the maintenance of the distal ends of the telomeres (Ahmed and Lingner, 2018). Breakage of the DNA backbone can also be provoked by ROS leading to telomere truncation when not repaired before

DNA replication (Aeby et al., 2016). ROS can also trigger premature abortion of the replication before reaching the end of the DNA template (Ahmed and Lingner, 2018).

The exact mechanism that increases the rate of telomere shortening by oxidative stress remains inconclusive. Several reasons have been postulated as to why telomeres are particularly vulnerable to oxidative damage. In vitro data has shown that oxidative stress can cause specific cleavage at polyguonosine sequences in the telomere sequence which may play an important role in increasing the rate of telomere shortening (Oikawa and Kawanishi, 1999). Telomeres were found to be extremely sensitive to the accumulation of ROS-induced 8-oxodG DNA-strand breaks (Zglinicki, Martin-ruiz and Saretzki, 2005). Single strand breaks to telomeric DNA was observed once disruption to TL maintenance and function occurs as a result of oxidative damage. Telomeres are repaired less efficiently in comparison to the rest of the genome and the repair is less proficient when damage is due to oxidative stress (Von Zglinicki, 2002). The high incidence of guanine residues in telomeric DNA sequences makes the telomere a more susceptible target for oxidative damage (Oikawa and Kawanishi, 1999). Finally, in conditions associated with oxidative stress, there is evidence to suggest that oxidative damage/ROS promotes telomere shortening and impairing telomerase activity thereby accelerating cellular senescence (Stefanidis et al., 2015)(Aeby et al., 2016).

Most evidence on oxidative stress and TL are from in vitro and animal models. There has been limited data published on true measurements of oxidative stress

markers in clinical studies. A study of 401 men with a mean age of 62.2 years revealed that urinary 8-epi-PGF_{2a} was inversely correlated with age adjusted TL, therefore increase systemic oxidative stress was associated with shorter TL consistent with findings in vitro to suggest oxidative stress accelerates telomere erosion (Demissie et al., 2006). A study of 58 healthy premenopausal women investigating psychological stress and cellular ageing showed that the group with shorter TL had higher F_2 Isoprostanes level, a reliable measure of oxidative stress (Epel et al., 2004). Most studies investigating biomarkers of oxidative stress in chronic disease has been in the field of diabetes. Results of increased oxidative stress in the form of 8-oxoguanine level are associated with shorter TL in diabetics in comparison to control group (Sampson et al., 2006). The reason for lack of studies directly measuring oxidative stress and TL may be due to the fact oxidative markers itself are challenging to assess reliably and it is difficult to control or account for the change of oxidative stress levels which may fluctuate over time with inter individual variation (Boonekamp et al., 2017). Therefore, this had led to the wider use of various indirect measures of oxidative stress i.e. a surrogate marker which than acts as a proxy for biomarkers of oxidative stress to provide a more reliable association.

There are more clinical studies investigating the effect of inflammation on TL. Wong et.al showed that increases in circulating levels of C-reactive protein (CRP) and serum amyloid A were associated with proportional decreases in peripheral blood mononuclear cells (PBMC) TL in a cohort of middle-aged workers exposed to occupational environmental pollution over 2 years of follow-up (Wong et al., 2014). In addition, cross-sectional analyses in a

population of 1,962 older adults ranging in age between 70 and 79 years showed that individuals with elevated circulating levels of either interleukin-6 (IL-6) or tumour necrosis factor-alpha had significantly higher odds for short PBMC telomeres, after adjustment for potential confounders (Donovan et al., 2011).

1.2.7 Telomeres and mortality

Individuals with shorter telomeres have a mortality rate nearly twice that of those with longer telomeres, with the loss in median survival associated with shorter telomeres was 4·8 years for women, and 4·0 years for men (Cawthon et al., 2003). A study of 143 healthy controls above the age of 60 years revealed that those with shorter telomeres in blood DNA had poorer survival, attributable, in part to a 3-fold higher mortality rate from heart disease (95% CI 1.36–7·45, p=0·008), and an 8·5-fold higher mortality rate from infectious disease (1·52–47·9, p=0·015) (Cawthon et al., 2003). This supports the hypothesis that telomere shortening in humans contributes to mortality in many age-related diseases. TL, when analysed as a continuous variable, was inversely associated with the age-adjusted mortality rate (r= -1·87, 95% CI –3·35 to –0·392, p=0·013) (Cawthon et al., 2003).

1.2.8 Telomere attrition

Humans have a relatively short TLs from 5-15kb and shorten at a rate of ~70bp per year (Canela et al., 2007) whilst mice who actually have longer TLs starting around 50kb but shorten at a rate of ~7000bp per year (Vera et al., 2012). Despite the fact humans have shorter TL they have a longer lifespan in comparison to mice who have a longer TL. This may be explained by the different rates of telomere shortening. Telomere shortening rate but not the initial TL alone is a powerful predictor of a species life span (Epel et al., 2009) (Vera et al., 2012) (Boonekamp et al., 2014)(Whittemore et al., 2019). These studies in animal models support the hypothesis that critical telomere shortening and the consequent onset of telomeric DNA damage and cellular senescence are a general determinant of life span.

Efforts have been made by several research groups to translate findings from animal models to humans. There have been 4 longitudinal studies investigating TL changes and lifespan/all-cause mortality as their primary outcome in adults (Martin-Ruiz et al., 2005) (Epel et al., 2009) (Bendix et al., 2014) (Yuan et al., 2018). The follow up duration on average for the study by Martin-Ruiz et.al was at least 3.9 years, Epel et.al was 2.5 years, Bendix et.al was 10 years and Yuen et.al was 7.4 years. Out of these studies, 3 studies failed to show any relationship between telomere attrition and mortality (Martin-Ruiz et al., 2005) (Bendix et al., 2014) (Yuan et al., 2018). The 4th study did find a relationship only for cardiovascular mortality and not total mortality (Epel et al., 2009).

The studies by Martin-Ruiz et.al and Bendix et.al had mean ages of 90 and 45 respectively. Baseline TL and TL attrition in both these mean age groups have been shown to be a poor predictor of mortality (Martin-Ruiz et al., 2005) (Bendix et al., 2014). The follow up duration in the study by Epel et.al was only 2.5 years which may not be long enough to demonstrate a difference in mortality. There have also been a few studies that had only investigated cardiovascular risk with longitudinal changes in TL. The Heart and Soul study had revealed a decrease in mortality in patients who had lengthened their telomeres among patients with stable coronary heart disease (Goglin et al., 2016). This study showed that during an average of 4.2 years of follow up, an increase in TL by 325 base pair was associated with a 24% reduction in mortality (HR 0.76, 95% CI 0.61-0.94; p=0.01), adjusted for age, sex, waist to hip ratio, exercise capacity, LV ejection fraction serum creatinine and year 5 TL (Goglin et al., 2016). Mortality was higher in patients with telomere shortening with a 32% adjusted increased risk of death versus telomere maintenance but this association was not significant (HR 1.32, 95% CI 0.84-2.08, p= 0.23) (Goglin et al., 2016). A another study had shown a more pronounced TL shortening in those with cardiovascular disease in comparison to non-cardiovascular disease patients (Benetos et al., 2018).

1.3 Telomeres in uraemia (CKD and RRT)

Chronic inflammation and oxidative stress accelerate cellular ageing through its effects on telomere biology. Uraemia promotes cellular senescence and premature ageing through toxic alterations in the internal milieu. The uraemic state in ESKD is considered a pro-inflammatory condition typically characterised by enhanced production of ROS (Jofré et al., 2006)(Libetta et al., 2011). The presence of inflammation and oxidative stress are common findings in patients with ESKD which has been widely addressed in the past. Increased oxidative stress of DNA is an established feature of uraemia (Tarng et al., 2000). Oxidative stress has been related to immune system dysregulation in patients with uraemia, indicated by increased biomarkers of oxidative stress and activation of circulating leucocytes (Sela, 2005). The repeated stimulation that cells; in particular PBMC are subjected to during haemodialysis (HD) as a result of repeated contact of blood to the extracorporeal circuit and dialyzer membranes may lead to the increased production of pro-inflammatory cytokines causing chronic inflammation associated with increased risk of mortality and morbidity (Pertosa et al., 2000). This may lead to irreversible cellular 'telomere-dependent' growth arrest called stress induced premature senescence (Bernadotte, Mikhelson and Spivak, 2016).

As outlined in the above paragraph chronic inflammation is related to telomere attrition and ageing. 'Inflammageing' is a condition characterized by elevated markers of inflammation which increases and accelerates ones susceptibility

to chronic morbidity, disability, frailty and premature death (Ferrucci and Fabbri, 2018). 'Inflammageing' in uraemia is the hallmark of premature ageing whereby the phenotypes exhibited are often observed in dialysis patients; which include accelerated atherosclerosis, protein energy wasting, sarcopenia, osteoporosis, frailty and cognitive dysfunction (Stenvinkel and Larsson, 2013). A reduction in lean mass also known as protein energy wasting and increase in fat mass known as sarcopenic obesity is associated with inflammation (Honda et al., 2007). Protein energy wasting associated inflammation is directly associated with increased comorbidity, diminished physiological, metabolic and immunological capacity which then leads to reduced survival in dialysis patients (Kim, Kalantar-Zadeh and Kopple, 2013). There are currently limited studies on the relationship of TL in the context of uraemia despite the wide consensus that uraemia is a recognised clinical model of premature ageing (Betjes et al., 2011)(Stenvinkel and Larsson, 2013).

Current evidence has revealed that during ageing, TL decreases more rapidly in the renal cortex than in the medulla, contributing to the cortical scarring and glomerular senescence observed in ageing kidneys (Yang and Fogo, 2010). In addition to age-dependent telomere shortening, factors such as oxidative stress and dysregulation of the renin-angiotensin system (RAS), commonly seen in uraemia can decrease TL and increase the intrinsic biologic age (Vasan et al., 2008).

Drugs which have revolutionised renal transplant has been shown to affect TL. Experiments on exposure of Cyclosporine A on HK-2 cells and primary

proximal tubular cells in vitro caused cell cycle arrest, inhibited DNA synthesis and led to a reduction in TL (Jennings *et al.,* 2007). These findings were confirmed by Koppelstaetter et.al. when investigating the effect of cyclosporine, tacrolimus and sirolimus on cellular senescence (Koppelstaetter *et al.*, 2018). The authors demonstrated reductions in TL and increases in hydrogen peroxide production; a marker of oxidative stress after cells were treated with cyclosporine but not with tacrolimus and sirolimus (Koppelstaetter *et al.*, 2018). The difficulties with in vivo studies revolve around the dosage of drugs used in the experiments and extrapolating the culture model to in vivo situations. Attempts to study immunosuppression and TL in vivo and in clinical studies have shown that TL assessment is useful to predict organ transplantation outcome. (Luttropp *et al.,* 2016)(Kłoda, Domański and Mierzecki, 2017). However, this has been complicated by inconsistencies is immunosuppression protocol, ischaemia reperfusion injury and the small size of the clinical studies to allow any conclusions to be drawn.

Several retrospective clinical studies have shown that TL is associated with kidney function, but this relationship is inconsistent. Data from a sub study of 620 patients with heart failure between ages 40 and 80 who participated in the metoprolol CR/XL Randomized Intervention Trial in Congestive Heart Failure (MERIT-HF) showed there were significant differences in TL and renal function defined by the MDRD equation after adjusting for age, gender and other baseline differences (van der Harst et al., 2008). The study revealed that TL positively correlated with renal function (van der Harst et al., 2008). In fact, the patients with estimated glomerular filtration rate (eGFR) a robust

marker of renal excretory function in the lowest quartile ratio had reduced TL in comparison to their controls which were not only age and gender matched but also matched on all the other baseline variables (r= -0.107, 95% CI 0.020 to 0.194, p = 0.017) (van der Harst et al., 2008). However, the cohort with the lowest renal function at baseline had an average eGFR of 42 ± 8.4 and serum creatinine (sCr) level of 137 [127-162] μmol/L. So, the results of the study cannot be applied to those with severe renal disease with or without the requirement for renal replacement therapy. This study was also unable to confirm if shorter TL is the cause or consequence in this population as this can only be determined from longitudinal data.

The Heart and Soul study; a longitudinal study of 954 participants revealed no significant correlation between telomere shortening and impaired renal function at baseline and over a 5-year period when age was taken into account (Bansal et al., 2012). The same study also suggested that accelerated telomere shortening is unlikely to contribute to the high burden of morbidity and mortality among patients with decreased kidney function independent of other risk factors such as advanced age (Bansal et al., 2012). However crucially, this study also excluded any participants with severe renal disease or individuals requiring renal replacement therapy.

Recent data from a longitudinal study of 80 healthy adults from China investigating TL using terminal restriction fragments (TRF) showed that TL was associated with renal function measured in the form of serum cystatin C

and eGFR (Zhang *et al.*, 2018). So, TL was negatively associated serum cystatin C and positively associated with eGFR ($p<0.001$). There was significant association between changes in TL i.e. telomere attrition over 3 years with serum cystatin C ($p=0.038$), but significance was lost after adjusting for age ($p= 0.0119$) (Zhang *et al.*, 2018).

Clinical studies have reported that patients with ESKD/CKD may have shorter TL and accelerated telomere shortening compared with the general population (Tsirpanlis et al., 2006)(Boxall et al., 2006). The first study looking into the role of TL in HD patients were published by Ramirez et al. This study consisting of 15 HD patients and 15 healthy controls and revealed that TL measured using FISH technique was reduced in dialysis patients and correlated with a raised CRP (Ramírez et al., 2005). Subsequently, a study of 43 HD patients and 23 healthy controls revealed no difference in TL between the 2 cohorts (Stefanidis et al., 2015). Another study using FISH technique in patients (n=137 patient with ESKD) with a mean age of 52.5 showed a significantly lower CD4 T cell TL compared with healthy controls (n=144) (Betjes et al., 2011). This study concluded that immunological age of ESKD patients was increased by 20-30 years compared with chronological age-matched health controls. A post hoc analysis from the MIMICK study, in HD patients showed that those with longer telomeres were younger and less inflamed with lower high sensitivity CRP (hs-CRP), IL-6 and 8-OH-dG levels (Carrero et al., 2008).

A retrospective case control study comparing 62 HD patients and 60 healthy age and sex matched adults showed that dialysis patients had shorter TL independent of age and sex [mean 8.8 kbp vs 10.5 kbp; $p<0.01$] when TL was measured with Southern Blot (Borras et al., 2012). A multivariate regression analysis of the HD subgroup did suggest that patients on active vitamin D treatment had greater TL than untreated patients (9.5 kbp vs 8.4 kbp; $p=0.003$) (Borras et al., 2012). In a Japanese observational prospective study, the lengths of telomeric G-tails and total telomeres were significantly shorter in 203 HD patients than in 203 control subjects that were both age and sex matched (Hirashio et al., 2014). Baseline data on TL in a study by Lutropp et.al. investigating telomere attrition a year post renal transplant did reveal that telomeres were longer in the non-chronic kidney disease (CKD) patients (median age of 58 years) in comparison to dialysis patients (median age of 55 years) supporting the theory that uraemia is a progeric state (Luttropp et al., 2016). In this study there were 63 patients in the control group and 49 patients on dialysis with an inter assay coefficient of variation (CV) of 0.32% for telomere and CV of 0.12% for 36B4. However, the data on TL was unadjusted for age and gender as the authors did not find any association between TL and these variables among healthy controls and dialysis patients. A study on 222 patients from an outpatient nephrology service in France of varying eGFR revealed that CKD was associated with premature senescence of the T cell compartment (Crépin *et al.*, 2018). Younger patients with CKD stage 4 and on dialysis had shorter TL than those with eGFR>60mls/min [1.116 ± 0.36 versus 0.786 ± 0.24 versus 0.976 ± 0.21 T/S ratio ($P< 0.001$), respectively] (Crépin *et*

al., 2018). Older patients with CKD Stage 4 and on dialysis had shorter TL than those with eGFR>60mls/min (0.986 ± 0.27 versus 0.826 ± 0.23 versus 0.886 ± 0.22 T/S ratio, p=0.045) (Crépin *et al.*, 2018). Young patients with ESKD has the same TL with older patients with eGFR>60mls/min (0.976 ± 0.21 versus 0.986 ± 0.27 T/S, p=0.810) (Crépin *et al.*, 2018). The authors reported a significant difference between TL in younger patients (age<65) and older patients (age>65) in 3 different groups; eGFR<60mls/min, CKD stage 4 and ESKD on dialysis (Crépin *et al.*, 2018). However, none of the data were adjusted for age and gender despite a significant difference between both these variables among the 3 group of interest.

A study comparing telomerase activity in 4 different groups of patients (n=20 in each group) with CKD revealed that the telomerase activity in PBMC were similar in CKD stages 2,3 and 4 (p>0.05) while in stage 5 CKD (20 HD and 10 peritoneal dialysis patients) it was significantly higher compared to other stages (p<0.0.5) (Kidir et al., 2017). The study found that telomerase activity was significantly lower in the healthy control group (n=20) compared to all other groups (p>0.05) independent of age, gender and inflammation (Kidir et al., 2017). However a more recent study of 224 patients showed no association between age and telomerase activity (Crépin *et al.*, 2018). This study also observed no difference between the 3 respective groups of interest; eGFR>60mls/min, CKD stage 4 and ESKD on RRT (HD, n=47 and PD, n=37) (Crépin *et al.*, 2018). Telomerase activity did not differ significantly in dialysis patients when grouped according to dialysis duration (Kidir et al., 2017).

Increased telomerase activity is synonymous with shortened TL. These findings suggest that telomerase activity could represent high level of cellular stress in dialysis patients and the increased activity is due to the repair and regulatory mechanism to maintain and prevent TL shortening. These findings support the notion that dialysis patients have an accelerated ageing in comparison to healthy controls based on telomerase activity.

Preliminary evidence that telomere shortening in vivo is linked with disease states in which oxidative stress plays a significant role comes from recent findings (Von Zglinicki, 2002). Increased levels of oxidative stress markers are common in the uraemic milieu and predicts poor outcome (Himmelfarb et al., 2002). Oxidative stress in association with chronic inflammation has been suggested for this increase in mortality (Von Zglinicki, 2002). Carrero et.al. was the first to show that shortened TL is associated with higher levels of DNA damage in the form of 8-OH-dG and raised mortality in HD patients independent of age and gender (Carrero et al., 2008). The study being part of participants from Mapping of Inflammatory Markers in CKD (MIMICK) study also showed that TL was negatively associated with hs-CRP, IL-6 and white blood cell (Carrero et al., 2008).

The cumulative exposure of oxidative stress in dialysis represented by telomere attrition has been studied in 70 HD patients which revealed that TL was inversely related to time since starting HD, also known as dialysis vintage ($r= -0.27$, $p= 0.02$) (Murillo-Ortiz et al., 2016). Another study of 43 HD

patients also showed that longer dialysis vintage was correlated with telomere shortening. (r=-0.332, p= 0.03) (Stefanidis et al., 2015). However, this association was largely due to 4 outliers with dialysis vintage between 4-6 years likely to have affected these findings. The authors did not report if the analysis was repeated by excluding the outliers. The largest study till date investigating the role of TL in dialysis patients was conducted by the Karolinska Institute in 175 HD patients as part of a post hoc analysis. The average mean dialysis vintage of patients in the study were 50 ± 60 months and there was no correlation between TL and dialysis vintage observed (Carrero et al., 2008). Conclusive data on this particular area has yet to be established as very limited studies are available on dialysis patients.

Cardiovascular disease is the leading cause of mortality in uremic patients with a 5-fold increased risk in comparison to the general population. Traditional risk factors commonly associated with cardiovascular mortality in general population do not discriminate well in dialysis patients. Telomere shortening in the general population is associated with increased risk of premature myocardial infarction, coronary heart disease and death (Cawthon et al., 2003)(Brouilette et al., 2007). Many cardiovascular research groups have suggested the use of TL as a novel predictor of cardiovascular outcomes in the general population.

Oxidative stress and chronic inflammation are well known risk factors of cardio vascular disease and play major roles in total telomere shortening in the

general population (Houben et al., 2008). Increased oxidative stress and inflammation have been widely associated in uraemia and these factors have been proposed to be a causative or associated factor that contributes to the raised cardiovascular mortality in patients with ESKD (Himmelfarb et al., 2002). A prospective observational study involving 203 Japanese HD patients examining the length of telomeric G-tails and total telomeres and subsequent cardiovascular events during a median follow-up period of 48 months revealed that the length of telomeric G-tail was associated with new onset cardiovascular events (Hirashio et al., 2014). These associations persisted despite adjustment for age, sex, diabetes mellitus, clinical history of cardiovascular disease, inflammation, use of vitamin D, and serum levels of phosphate and intact parathyroid hormone. Similar findings were not seen with total telomeres in this study. This was the first study to report the findings of telomeric G-tail lengths as an independent predictor of new onset cardiovascular events in HD patients. Shortening of telomeres have also been associated with significant increase in left ventricular mass in HD patients ($r=0.40$, $p=0.02$) (Murillo-Ortiz et al., 2016). This study also revealed that left ventricular mass index was higher in the group of patients with raised ferritin and concluded that iron induced oxidative stress could play a fundamental role in their findings (Murillo-Ortiz et al., 2016).

Currently, the relationship between ESKD and TL; and mortality has not been well explored. Most studies exclude patients on renal replacement therapy which makes it impossible to evaluate any association between ESKD and telomere biology. However in vivo and in vitro studies as well as population

data has shown the relevance and importance of investigating telomere biology in patients on renal replacement therapy. Telomere biology could be a contributing or causal factor in mortality outcomes in the uraemic population and could explain the markedly increased risk of cardiovascular mortality in patients with ESKD.

1.3.1 Telomere Attrition in uraemia (Chronic Kidney Disease and Renal Replacement Therapy)

Longitudinal studies in animal models and non-CKD/RRT patients have helped us understand the importance of TL attrition and the role it plays in lifespan/mortality. However, the role of telomere attrition in uraemia is not well understood as there are only a handful of published work in this area. The first and only study thus far investigating telomere attrition in dialysis patients (n= 59) did report that dialysis patients with longer TL at baseline had increased telomere attrition (Kato et al., 2016) as seen in animal models and studies in healthy and non-CKD/RRT adults (Refer to Section 1.2.8 on telomere attrition). The study was unable to draw any conclusion on mortality due to the small number of study participants and low number of events (Kato et al., 2016). Luttropp et.al had investigated the role of telomere attrition post renal transplant in 47 patients (median age of 45 years) in comparison to 49 patients (median age of 55 years) on dialysis and 63 patients with no renal disease (Luttropp et al., 2016). Over 12 months, dialysis patients had showed a small but significant decrease in TL (0.88 to 0.85 T/S; p=0.03) whilst patients

who underwent kidney transplant had surprisingly a significantly greater telomere attrition (1.02 to 0.84 T/S; $p<0.001$) (Luttropp et al., 2016). The authors had hypothesized that TL attrition would be less after renal transplant due to normalization of pro-oxidative, pro-inflammatory and hyperhomocysteinaemic uraemic milieu as renal transplant is associated with better long-term survival in comparison to dialysis treatment (Luttropp et al., 2016). One possible reasons for these unexpected findings may be attributed to different rates of attrition in different age groups, the effect of immunosuppression e.g. mycophenolate mofetil (Luttropp et al., 2016) and anti-thymocyte globulin (Crepin et al., 2015), increased BMI post-transplant due to steroid use and the probable increase in telomere attrition associated with major surgery which may improve with time. However, bearing in mind that the authors did not adjust TL findings with age and gender as there was no correlation between these three variables in the study cohort. This highlights the importance for the need of further studies to investigate these findings in different age groups and longer duration of follow up as little is known about the dynamic change and regulation of TL over time and consequences in uraemia.

1.4 DNA methylation and epigenetics

DNA methylation (DNAm) is another promising biomarker that has gained recognition as an ageing molecular marker. It is an epigenetic marker of the genome linked to gene expression/regulation and is involved in the development process of various eukaryotes. Epigenetics is defined as a functionally relevant structural and chemical change of DNA and/or associated regulatory proteins without any change to the nucleotide sequence (Felsenfeld, 2014). These changes alter the availability of DNA to bind proteins that can either induce or repress gene transcription.

DNAm involves the addition of a methyl (CH_3) group to the carbon 5 position of the cytosine ring of the DNA resulting in an end product known as 5-methlycytosine (5-mC). This process subsequently modifies the gene and its expression thereafter. DNAm is a heritable and modifiable factor affecting gene expression. 5-mC is found in approximately 1.5% of human genomic DNA (Lister et al., 2009). The addition of methyl groups is catalysed by DNA methyl transferases (DNMT); which consists of a group of enzymes; DNMT1, DNMT3a and DNMT3b. DNMT1 is involved in the maintenance of the methylation pattern in the genome by replicating the hemimethylated CpG sites (Vilkaitis et al., 2005). Whilst DNMT3a and DNMT3b are de novo methlytransferases (Okano et al., 1999). Transcription factors and histones are known to direct DNMTs to specific genomic regions, these mechanisms are poorly understood.

Passive DNA demethylation which is thought to occur during replication in the absence of a maintenance mechanism. Active DNA demethylation is a process whereby the methyl group is removed or modified from the 5-mC section of the DNA aided by an enzymatic process. It is only in recent years that series of discoveries have led to a better understanding of the mechanism involved in active DNA demethylation which involves the ten-eleven translocation family of enzymes. Ten eleven translocation (TET) dioxygenases are thought to play an important role in DNA demethylation through conversion of 5mC to 5hmC (Kohli and Zhang, 2013).

In the context of gene promoters, hypomethylated CpGs are associated with active, constitutively expressed genes. Hypermethylation of gene promoters which have high densities of CpG sites (28 millions of CpG sites in the human genome), known as CpG islands, leads to chromatin compaction an associated with consequent loss of gene expression which is a critical mechanism for control of expression of imprinted genes (Bird, 2002).

1.4.1 Factors associated with DNA methylation

The epigenetic profile has shown that DNA methylation is relatively dynamic and can be influenced by many factors. Twin studies have discovered that age, gender, genetics, habits and environmental factors affect DNA methylation. (Fraga et al., 2004)(Tan et al., 2016) . Measures of obesity such as body mass index (BMI) and waist circumference are shown to be associated with accelerated epigenetic ageing (Horvath et al., 2014). DNA methylation may also be influenced by smoking (Park et al., 2018) and stress (Matosin, Cruceanu and Binder, 2017). However, such a correlation was not observed in other studies (Sillanpaä et al., 2018).

1.4.1.1 DNA methylation profile and gender

It is generally assumed that the age-related changes in DNA methylation are largely similar between males and females. However, a study by Masser et.al. showed that methylation does not differ between gender at young age but an age related change occurs in one sex termed ‘sex divergence’ (Masser et al., 2017). A meta-analysis of four cohorts; the Lothian Birth Cohorts of 1921, and 1936, the Framingham Heart Study, and the Normative Ageing Study showed that women had significantly lower Δ age (DNAm age minus chronological age) estimates than men i.e. men older than women (Marioni, Shah, McRae, Chen, et al., 2015). Similar findings were also seen by Hannum et.al. when he built his quantitative model of ageing with 656 individuals

(Hannum et al., 2013). A recent systematic review investigating the association between the epigenetic clock and increased risk of death showed no clear difference across gender (Fransquet et al., 2019).

1.4.1.2 DNA methylation in chronic disease states

The pathogenic role of DNAm in diseases is diverse. There is mounting evidence to suggest that DNAm has an important regulatory role in the development and progression of common diseases, with a systematic review showing that an increase in DNAm age predicted the future risk of diseases (Fransquet et al., 2019).

A link between DNAm and cancer was first described in 1983 when cancer cells were found to be hypomethylated in comparison to healthy cells (Gama-sosa et al., 1983). However, this hypothesis has evolved with further research suggesting that both hypomethylation and hypermethylation are observed in virtually all types of cancer. However, the pattern of DNAm differs in different types of cancer and different specimens. Recent discoveries in cancer epigenetics has led to classification of epigenetics into 3 different classifications; epigenetic modifiers that directly modify the cancer epigenome and are frequent targets of mutations and epimutations; epigenetic mediators that drive the tumour or its progenitor cells towards a more stem-like state; and epigenetic modulators that transmit environmental signals to epigenetic modifiers (Feinberg, Koldobskiy and Göndör, 2016).

Aberrant DNA methylation is not only associated with various cancers but it's may have a role in other chronic conditions including autoimmune diseases, neurodegenerative conditions and metabolic disorders (Jin and Liu, 2018). Linking DNA methylation status at specific gene regions with chronic diseases is an active field of research. With the hope that this knowledge can be used to identify new risk factors and provide more sensitive markers for diagnosis, prognosis and potential treatment.

Another area that has gained wide recognition in the field of DNA methylation is ageing. There is evidence to suggest that DNA methylation throughout an individual's lifespan exhibits a strong correlation with age and age-related outcomes. The first evidence that ageing might have an effect on DNA methylation was reported in 1973 whereby total levels of 5-mC in DNA isolated from various tissues of albino male rats reduced with age; 1, 12 and 28 months (Vanyushin et al., 1973). Subsequent studies in animal models and cultured human cells confirmed these findings and reported the rate of loss of DNA 5-mC residues as an inverse relationship to life span (Wilson et al., 1987). The relationship between DNA methylation and ageing is extensively discussed in below.

1.4.2 DNA methylation and ageing

There are 6 types of potential biological age estimators which are epigenetic clocks, TL, transcriptomic- based, proteomic- based and metabolomic-based estimators and composite biomarkers. DNAm age is often referred to as 'epigenetic clock'. A recent review concluded that the epigenetic clock is the most promising molecular estimator of biological age (Jylhävä, Pedersen and Hägg, 2017).

A small fraction (~2%) of the CpG sites show age related change either hypermethylation or hypomethylation. DNAm has an inverse relationship with age in humans (Langevin et al., 2011)(Steves, Spector and Jackson, 2012). DNAm predicts biological age more efficiently than chronological age (Xiao, Wang and Kong, 2019). The difference between the DNAm age and chronological age provides the rate of epigenetic age attrition. Accelerated epigenetic ageing is when estimated age is higher is than the chronological age. Therefore, those with an epigenetic change age that is older than expected based on their chronological age are described as exhibiting positive epigenetic age acceleration whilst the reverse situation would be described as negative age acceleration. DNAm age can predict life expectancy because it has been shown to predict all-cause mortality independently of common risk factors (Bressler et al., 2016)(Chen et al., 2016)(Lin et al., 2016).

An ageing phenotype occurs as a result of cellular senescence which refers to the process of cell proliferation arrest which is irreversible. One of the contributing factors to cellular senescence is stress which leads to DNA damage. Cellular senescence shares epigenetic similarities with ageing. Studies have shown that the process of replicative senescence is associated with loss of DNA methylation as seen in ageing (Wilson and Jones, 1983). In fact, senescence can be induced with DNA methylation inhibitors in vitro (Vogt and Haggblom, 1998). Recent analyses of primary endothelial cells have demonstrated an increased epigenetic age with senescence in an age dependent manner (Lowe, Horvath and Raj, 2016).

In general, cellular senescence and epigenetic ageing are distinct phenomena though seem to converge under the same circumstances. Even though senescent cells exhibit methylation changes of some specific CpGs, these are in general have not been similar sites noted in age-related CpGs (Franzen et al., 2017).

Many efforts thus far have been spent in identifying dysregulation of the epigenetic landscape which has now been recognised as a hallmark of the ageing process. Changes to the epigenetic process occurs throughout the entire lifetime of an individual. In humans, DNAm changes start early in life as demonstrated by longitudinal studies of infant's blood where increased levels of 5mc were shown to have already occurred during the first year of life during a 5 year follow up period (Herbstman et al., 2013) (Martino et al., 2013). In vitro studies have shown that senescent fibroblasts and various tissues from

aged mice exhibited low levels of 5mC (Wilson and Jones, 1983)(Wilson et al., 1987).

The first genome wide analysis focusing on 5hmC in ageing was performed on human mesenchymal cells that revealed equal amounts of both 5hmC gain and loss. The epigenetic theory is currently based on 2 theories as described below:

a) Epigenetic drift

This theory is defined as the general decrease in DNAm or hypomethylation whereby the baseline DNA methylation progressively diverges during ageing with increases in variability of methylation patterns with age (Fraga et al., 2004). Epigenetic drift leads to inter individual epigenetic divergence with increasing age as a result of lifelong accumulation of epimutations. Evidence for this comes from monozygotic twin studies (Fraga et al., 2004) (Jones, Goodman and Kobor, 2015) which have shown that twins with similar epigenome at birth become more discordant as they age. This may be due to influence of environmental factors and intrinsic factors.

b) Epigenetic clock theory

This theory is described as locus specific DNA methylation events in gene-associated regulatory regions which suggest a programmed epigenetic reconfiguration during ageing (Horvath, 2013). Several studies have supported epigenetic clock theory showing an age associated epigenetic programme

which consists of DNA methylation changes at specific CG sites that are reproducible across individuals and tissues. These studies led to the development of two predictive mathematical models of biological age based on ageing associated site-specific DNA methylation changes (Hannum et al., 2013) (Horvath, 2013). The models were basically age predicting tools that were developed after interrogating DNAm states at various CG sites. Hannum's clock had assessed 71 CG sites from whole blood samples of more than 650 individuals (Hannum et al., 2013). The model had a 96% correlation with a margin of error of 3.9 years. The Horvath's clock had assessed 353 CG sites from more than 7800 samples consisting of 51 different cell and tissue types. Horvath confirmed that the hypermethylated sites with ageing were compatible with a broad range of tissues with a 96% correlation with a margin of error of 3.6 years (Horvath, 2013). Horvath had determined that the ageing rate of the methylome follow a logarithmic increase until adulthood following by a linear relationship thereafter (Horvath, 2013). Both these models allow the calculation of an individual's epigenetic age despite having only 6 CpG sites in common (Chen et al., 2016). The predicative accuracy of the Hannum clock, which was developed using blood samples, declines when applied to other tissues. In contrast, the Horvath clock performs well with a variety of tissues; however, the accuracy of the Horvath clock in predicting age is less for skeletal muscle, heart, and breast. Both Horvath and Hannum clocks are models based on chronological age which may be account for strong correlation between these epigenetic clocks and chronological age. Δ age (predicted age or also known as DNAm age minus chronological age) correlates negatively with age. Horvath's group subsequently developed an

epigenetic marker which would be a better predictor of biological age by incorporating other clinical measures (e.g. albumin, creatinine, c-reactive protein etc) in addition to chronological age. The resulting Pheno-Age clock has 41CpG sites it shared with Horvath's data and 6 similar sites with Hannum (Levine et al., 2018). Pheno-Age remains highly correlated (r^2=0.92) with chronological age and is a better reflection of biological age and mortality (Levine et al., 2018).

The revelation of the epigenetic theory has led to increasing efforts to determine any association between DNAm and ageing. Research has shown that epigenetic changes affect all cells and tissues over the lifespan (Talens et al., 2012). Early studies showed that global DNAm decreases with age, however subsequent studies have shown that ageing is associated with mainly hypermethylation of several genomic loci (Gensous et al., 2017). Studies have concluded strong links between DNA methylation, age acceleration, increased risk of cardiovascular disease and mortality. There has also been promising data from animal studies. Petkovic et.al. reported that the epigenetic clock accurately predicted chronological age of mice but none of the CpG sites matched the site from either Hannum or Horvath (Petkovich et al., 2017). Despite the fact that no CpG sites matched between the mice epigenetic clock and the human epigenetic clock there has been promising data on anti-ageing therapy in the animal models. Studies have shown that a caloric restriction diet lowered the epigenetic age of mice by 20% compared to the chronological age (Petkovich et al., 2017) (Wang et al., 2017). Research assessing the role of

rapamycin in animal models had also revealed that mTOR inhibitor had increased the lifespan of a variety of mouse models (Wang et al., 2017).

Interest in the role of DNAm and ageing increased following the development of the epigenetic clock by Horvath and Hannum following interrogation of various CpG sites to predict chronological age. These studies only looked into CpG sites. However, despite efforts of these groups, it is still difficult to confidently ascertain if methylation changes of CpGs are the driving force of ageing or the cause of consequence of the phenotype as the exact mechanism by which DNA methylation regulates ageing is still poorly understood. Further studies in this area are vital to gain insight into DNA methylation genome changes at specific sites with age across different cohorts.

1.4.3 DNA methylation and mortality

The rate of ageing, susceptibility to diseases and death differs from person to person. The underlying explanation to explain this variation has been the backbone of research in the area of DNAm. A meta-analysis by Marioni et.al. reports that the difference between DNAm predicted age and chronological age (Δage) using two sets of epigenetic markers (Hannum and Horvath's epigenetic clock) were associated with an increased risk of mortality in four independent cohorts of older individuals with a mean age of 72 years (Marioni, Shah, McRae, Chen, et al., 2015). This association was independent of life-course predictors of ageing and death such as possession of the e4 allele of

APOE, education, childhood IQ, social class, diabetes, high blood pressure, and cardiovascular disease (Marioni, Shah, McRae, Chen, et al., 2015). The authors also showed that there was no clear association between these factors and Δage, supporting the hypothesis that the epigenetic clock and DNA methylation Δage is predictive of human mortality, after accounting for known risk factors.

A recent systematic review found that a 5 year increase in DNAm age was associated with up to a 15% increased risk of all-cause mortality (Fransquet et al., 2019) In fact the primary finding of this systematic review of 11 studies (278490 participants and 10233 deaths) support an association between accelerated DNAm age and an increased risk of all-cause mortality in particular when the Hannum epigenetic clock was used (Fransquet et al., 2019). Ten out of the 11 studies in this systematic review had independently found that a higher biological age relative to chronological age is predictive of time to death, cancer-related, cardiovascular disease related or all-cause mortality (Fransquet et al., 2019). Two meta-analysis; one with 13 studies of n=13089 and another with 4 studies of n=4658 reported a significant association between an increase in DNA methylation age and mortality risk (Marioni, Shah, McRae, Chen, et al., 2015)(Chen et al., 2016). Marioni et.al. revealed that a 5-year mortality was higher with the Hannum and Horvath Δ age at 21% and 11% when corrected for age and sex (Marioni, Shah, McRae, Chen, et al., 2015). Horvath's calculator is better for development stages e.g. puberty and menopause whilst Hannum's clock may be a better reflection of later life disease states and mortality (Levine et al., 2018)

1.5 The importance of DNA methylation and uraemia

DNAm has been associated with different diseases (Laird, 2003)(Feinberg, 2007). Research into the role of DNAm has been limited in dialysis cohorts. There has been some evidence in the role of DNAm in the context of uraemia; mainly on cardiovascular risk and inflammation that has shed some light on epigenetic DNA modifications in this cohort (Stenvinkel et al., 2008).

Reports from ESKD cohorts have shown that dialysis patients have an increased risk of cardiovascular disease; at least 10-30 times higher than the general population with a 23% mortality. This increased risk cannot be explained by the conventional risk factors alone and has been linked to elevated oxidative stress levels, persistent inflammation and hyperhomocysteinaemia. Therefore, exploring the role of the epigenetic clock in uraemia would seem to be naturally the next step in progressing forward in renal research.

A study by Stenvinkel et.al. showed that global DNA methylation was associated with both all-cause mortality and cardiovascular mortality even when adjusted for age, CVD, diabetes and inflammation in dialysis patients (Stenvinkel et al., 2007)(Stenvinkel and Ekström, 2008). The study highlighted that global DNA hypermethylation was the strongest independent risk factor for mortality from CVD adjusting for age and previous cardiovascular disease.

The study also revealed a strong association between markers of inflammation and oxidative stress (IL-6 and 8-OHdG) and global DNA hypermethylation (Stenvinkel et al., 2007). There was only a weak significant correlation between DNA methylation status and hyperhomocysteinaemia in this study though (Stenvinkel et al., 2007). Another smaller study of 20 healthy controls and 20 dialysis patients failed to show any difference between global DNA methylation between both groups (Hsu et al., 2012). However, the study did reveal a reduction in DNMT expression (an enzyme group responsible for the transfer of methyl group to the 5-position of cytosine residues in DNA and to establish and maintain genomic methylation) in HD patients (Hsu et al., 2012).

Subsequent efforts have taken place to identify probable gene promoter regions which could account for these findings. A study of DNA methylation status of the MTHFR promoter region showed a significant increase in the frequency of the MTHFR methylation status among patients with ESKD in comparison to healthy controls (Ghattas et al., 2014). MTHFR gene has been associated with hyperhomocyteinaemia which has been associated with increased rates of vascular disease. Findings from these studies have given researchers a glimpse of potential and importance of the epigenetic clock especially in uraemia though development of this field in nephrology is still premature.

1.6 Frailty

1.6.1 What is frailty- concept and definitions?

Frailty is a biologic syndrome where there is a decreased reserve and resistance to stressors, resulting from cumulative declines across multiple physiologic systems, which in return causes vulnerability to adverse outcomes (Fried et al., 2001). Frailty is often recognised as a phenotype that quantifies the functional loss that results during the ageing process. Frailty is also a multidimensional construct reflecting the decline in health and function observed in the elderly, ultimately resulting in increased risk for adverse health outcomes, falls and disability, hospitalization, institutionalization and death (Clegg et al., 2013).

Frailty is not synonymous with either comorbidity or disability, but there is an overlap among these 3 phenotypes whereby comorbidity is an aetiologic risk factor for, and disability is an outcome of frailty (Fried et al., 2001). Frailty was defined by Fried et al based on 5 components; grip strength, walk speed, depression scale, weight loss and physical activity. There have been various frailty screening tools that has been developed to detect frailty but there is no gold standard identified thus far.

1.6.2 Why is frailty an important?

Old age itself does not define frailty but prevalence of frailty increases with age. In the Cardiovascular Health Study, involving 5317 individuals who were 65 years and older revealed that frailty increased with age (Fried et al., 2001). Frailty was also higher in females than male (Fried et al., 2001) (Woo et al., 2008). Individuals with intermediate frailty (i.e. pre –frail with scores of 1 or 2) were more than twice the risk of becoming frail over 3 years relative to those with no frailty characteristics at baseline (Fried et al., 2001). Presence of frailty is also associated with several adverse health outcomes such as functional decline [worsening mobility, activities of daily living (ADL) disability, recurrent falls, hip and non-spine fractures], hospitalization, and death (Fried et al., 2001). Frailty is a strongly associated with impaired physical ability, poorer quality of life and increased morbidity and mortality. The International Task Force on Nutrition and Ageing concluded that gait speed (one of the components of frailty) is strongly associated with adverse outcomes, including fall and hospitalization in non-renal patients (Abellan Van Kan et al., 2009).

In men, higher frailty index was positively associated with mortality after adjusting for age, while the association was not significant in women (Woo et al., 2008). PBMC telomeres were shorter in sarcopenic subjects (T/S= 0.21; 95% CI: 0.18–0.24) relative to non-sarcopenic individuals (T/S= 0.26; 95% CI: 0.24–0.28; p=0.01), independent of age, gender, smoking habit, or

comorbidity (Marzetti et al., 2014). A recent overview did suggest that TL may provide a better and more accurate assessment and prediction of frailty in elderly population (Lorenzi et al., 2018). A recent study has shown that frailty can be modified by exercise and nutrition; reversing the frailty state to non-frail (Michel, Cruz-Jentoft and Cederholm, 2015). Understanding these associations can help recognise elderly individuals at risk for adverse outcomes and in return improve care for this vulnerable subset of patients as well as exploring means of reversing frailty.

1.7 Frailty phenotype in uraemia

The presence of chronic diseases was associated with increased frailty. Frailty can be caused by ageing per se, advanced CKD or both. Fried et al. described an association between CKD on one hand, and the development of functional impairment on the other. This was further reinforced by Roderick et al. revealing that measures of morbidity and functional impairment increased as renal function worsened (Roderick et al., 2007).

There are various metabolic alterations associated with uraemia which inevitably impact and affect frailty in patients with ESKD. Uraemia induced alterations such as increased energy expenditure, inflammation, acidosis, multiple endocrine perturbations that render a state of hypermetabolism leading to excess catabolism of muscle and fat are factors that have significant impact on protein energy wasting and subsequently contribute and affects a

dialysis patient's frailty level and accelerates ageing. Frailty was found to be predictive of poor outcomes among patients with ESKD. The validity of the frailty index was supported by its association with mortality (Mitnitski et al., 2002), and health outcomes such as hospitalization, functional and cognitive decline and was also influenced by social determinants (Woo et al., 2008).

Frailty was highly prevalent in patients with ESKD and substantially greater in elderly and near-elderly ESKD patients (Johansen et al., 2007). The prevalence of frailty in the older adult population is reported to be 11%, whereas the prevalence of frailty has been reported to be greater than 60% in dialysis-dependent patients (Johansen et al., 2007). Increased mortality and higher risk of combined outcome of death or hospitalization was also seen independent of age among frail dialysis patients in the dialysis Wave II Study (adjusted HR 2.24, 95% CI 1.60-3.15) (Johansen et al., 2007). Frail patients were more likely to be hospitalized for any reason and have increased mortality rate (HR1.90;95%CI 1.67 to 2.17), a finding that persisted after adjustment for multiple potential risk factors for hospitalization (adjusted HR 1.56; 95% CI 1.36 to 1.79) (Johansen et al., 2007). Even after adjustment for age, gender, race or ethnicity, body size, dialysis modality, comorbidities, serum albumin, and other factors, frailty remained strongly associated with mortality (adjusted HR 2.24; 95% CI 1.60 to 3.15) (Johansen et al., 2007). Multivariable logistic regression analysis of the dialysis Wave 11 study also suggested that older age, female sex and HD (rather than PD) were independently associated with frailty (Johansen et al., 2007). The study also revealed that among HD patients, those with a permanent vascular access (fistula or graft) were less likely to be frail,

though these finding are open to confounding of access choice based on physician assessment of frailty (hazard ratio HR 0.71; 95% confidence interval CI 0.51 to 0.98).

There was a lack of interaction between age and frailty in ESKD cohort suggesting frailty is relevant even in the younger patient (Johansen et al., 2007). In the Dialysis Wave 11 Study a significant proportion of patients in the younger age groups were also frail with 44% of these patients being younger than 40 years of age and more than half of the frail patients were aged between 40 to 50 years.

Shlipak et al. showed that CKD is associated with a greater prevalence of both frailty and disability in the elderly population, with the prevalence of frailty and disability being 15 and 12%, respectively, in elderly with CKD, versus only 6 and 7% in the elderly with normal kidney function, respectively (Shlipak et al., 2004).

Frailty also increases over time in the absence of any intervention. There have been a few studies evaluating the rate of change in frailty status over a period of time but there have been no prospective studies looking into effect of intervention on frailty on a longitudinal basis in dialysis patients. The investigators of a 2 year follow up study (ACTIVE/ADIPOSE study) on frailty among 771 patients showed that components of frailty did not change to the same degree or in the same direction over the follow up period (Johansen et al., 2019). The authors revealed that the 5 components of frailty; physical

activity, gait speed and to a lesser extent grip strength declined over time but exhaustion was relatively stable and the odds of fulfilling the weight loss criteria decreased over the study period (Johansen et al., 2019). All the 5 components except physical activity were associated with mortality independent of all the other components but the use of all 5 components in the form of a model provided the most useful information (Johansen et al., 2019).

These findings have led researchers to believe that there is a higher proportion of frail patients in the ESKD cohort in comparison to those without kidney disease suggesting that ESKD may be a major contributor to the development of frailty (or vice versa), such that it can occur in patients who are younger than those who are typically expected to be frail. These studies have also paved the way into using individual frailty components to help stratify risk of adverse outcomes which cannot be provided by usual clinical measurements.

1.7.1 Physical Frailty Phenotype/Fried or Hopkins Frailty Phenotype

The Physical Frailty Phenotype, also commonly known as the Fried Frailty phenotype is the most cited frailty tool in the research literature and validated in dialysis cohorts (Buta et al., 2016). The tool defines the frailty phenotype as scoring 3 or more of the 5 frailty criteria listed below while pre-frail is defined as a score of 1 or 2 of the characteristics and not frail as scoring 0.

a) Weight loss (more than 10 pounds unintentional)
b) Exhaustion or fatigue
c) Muscle Weakness (assessed by grip strength)
d) Slow walking speed (gait speed to walk 15 feet)
e) Low physical activity

The Fried frailty phenotype is an ideal tool to be used in the dialysis cohorts as each of the frailty component represent and reflect changes often seen among dialysis patients due to the pathophysiology of the disease condition. The frailty components measured affect and regulate each other. Alteration to one component may impact another component. Often there is a huge overlap and dependence between the frailty components measured, with a bidirectional relationship.

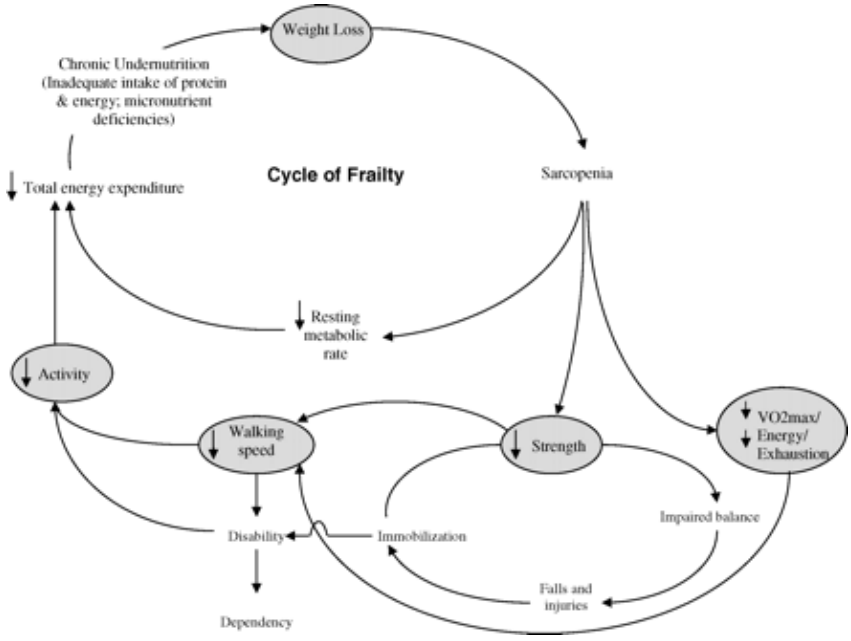

Figure 7: Cycle of frailty. Reproduced with permission. (Xue et al., 2008)

1.7.2 Factors associated with frailty in uraemia

There are various factors that contribute to frailty in the elderly population and more so among elderly patients with ESKD as listed below.

a) Increase inflammatory markers

Chronic inflammation is a hallmark finding in dialysis patients and is the main feature of uraemic phenotype associated with increased cardiovascular disease and protein energy wasting. It is a known predictor of poor outcome in ESKD. C-reactive protein and interleukin-6 (IL-6) are often raised in ESKD and associated with frailty (Cesari et al., 2004)(Cohen, Harris and Pieper, 2003) (Hubbard et al., 2009). IL-6 seem to be a robust predictor of comorbidity and all-cause mortality

in CKD stage 5 (Sun et al., 2016). All the 5 components measured in the frailty component is a direct and indirect measure of inflammation observed in ESKD cohort (Wang et al., 2012) (Bossola et al., 2015).

b) Anaemia

Anaemia is often observed in dialysis patients and is managed with iron and erythropoietin The Women's Health and Ageing Studies I and II (WHAS) identified anaemia as a risk factor for frailty (Chaves et al., 2005). Exhaustion is associated with anaemia in dialysis patients which subsequently leads to low physical activity and this can affect walking speed.

c) Poor nutrition, weight loss and sarcopenia

Anorexia is common among patients on dialysis with a prevalence of up to 50% (Carrero, 2009). The cause of weight loss in dialysis patients are multifactorial. There is true undernutrition due to insufficient food intake resulting from poor appetite and various dietary restrictions observed by patients on dialysis and pathological nutritional deficiencies due to uraemic toxins, chronic inflammation, acidosis, altered hormone function and the process of dialysis itself which results in loss of amino acids stimulating muscle breakdown. Weight loss is significantly more pronounced among dialysis patients and leads to an accelerated loss of muscle mass and strength causing sarcopenia. Sarcopenia causes reduced hand grip strength, affects speed of walking, leads to physical inactivity and causes exhaustion.

Sarcopenia reflected by slow gait speed and low muscle strength from the ACTIVE/ADIPOSE study of prevalent HD patients showed that these frailty components were associated with mortality even after adjustments for muscle size and other confounders (Kittiskulnam et al., 2017). Diminished hand grip strength was a significant predictor of death in dialysis patients (Yoda et al., 2012) and independent risk factor for all-cause mortality (Vogt et al., 2016).

d) Hormonal changes

The kidneys play a major role in endocrine function. ESKD is associated with impairment of excretion, synthesis and functionality of many hormones including insulin, insulin growth factor (IGF), growth hormone (GH), testosterone and thyroid function. These hormones are implicated in malnutrition, loss of muscle mass and closely associated with quality of life. Giving GH to elderly patients on HD improved muscle performance, suggesting a role of GH in preserving muscle function. Studies in CKD patients have shown that endogenous testosterone was an independent determinant muscle mass and muscle strength measure with hand grip strength (S. et al., 2013). Randomized intervention studies with androgen replacement in patients with renal dysfunction did show significant improvement in muscle mass and nutrition (Johansen, Mulligan and Schambelan, 1999). Therefore, the indirect effects of hormonal changes in dialysis patients can be reflected by the 5 components of frailty.

e) Bone mineral density

Dialysis patients suffer from secondary and tertiary hyperparathyroidism and achieving adequate bone mineral density are key goals in managing a dialysis cohort. Bone mineral diseases in dialysis patients are associated with disturbance of phosphate and calcium homeostasis and changes in key regulators such as parathyroid hormone and fibroblast growth factor 23. Alterations in these parameters will lead to renal osteodystrophy with loss of bone mineral density, increased risk of osteoporosis, fractures, falls and premature vascular calcification. Patients on dialysis have been observed to have a 2 to 4 fold increase in hip fracture rate (most extensively studied fracture type in ESKD patients) in comparison to the general population (Alem et al., 2000) (Arneson et al., 2013). Poor mineral bone density/osteoporosis is often represented by weak hand grip strength, reduced physical activity and may inadvertently affect walking speed which is part of the frailty components.

f) Depression

Comorbid depression is common in dialysis patients with studies suggesting that up to 1/3 of all patients on dialysis have significant depression (Palmer et al., 2013). Depression in dialysis patients is associated with fatigue, loss of appetite and difficulty in sleeping. Some of the health risk observed in dialysis patients with depression include inability to adhere to the prescribed dialysis regime, diet, medication and self-harm (DiMatteo, Lepper and Croghan, 2000). There is significant evidence to demonstrate that higher negative

perception of illness to be associated with higher depression scores and lower quality of life. Progression of depressive symptoms is associated with the onset and progression of frailty in non-dialysis when using the Centre for Epidemiological Studies Depression Scale (Ní Mhaoláin et al., 2012). Studies have revealed an association between increased markers of inflammation (c-reactive protein and interleukin 6) in dialysis patients with exhaustion and depression (Wang et al., 2012) (Bossola et al., 2015). The exhaustion/fatigue, unintentional weight loss and low physical activity component from the Fried frailty phenotype can also signify depression. Depression correlates with both hospitalization and mortality rates and has been associated with frailty (Lopes et al., 2002).

1.8 Telomere biology and DNA methylation

There is emerging evidence to suggests that epigenetics regulates telomere dynamics in adults (Buxton et al., 2014; Wong et al., 2014). Studies in animal models described the role for mammalian DNA methyltransferases in TL control which demonstrated a previously unappreciated role for DNAm in telomere maintenance (Gonzalo et al., 2006). Subsequent studies in human adults found that there was an increase in global DNAm at the proximal subtelomeric regions which was associated with an increased in telomerase activity which is involved in telomere maintenance (Ng et al., 2009) Both global DNAm and TL are inversely associated and involved with ageing process and disease vulnerability. TL exhibits weak negative correlations with

epigenetic age acceleration defined by Hannum's clock and DNAm PhenoAge (based on 513 CpG sites and takes into account 42 clinical biomarkers and age) and this is probably due to age-related changes in blood cell composition such as the decline in naive T cells often affecting TL (Chen et al., 2017) (Levine et al., 2018). Both DNAm age and TL are associated with age and mortality independent of each other (Marioni et al., 2016). Data from the Lothian Birth Cohorts 1921 and 1936 revealed that the variance in age with TL was 6.6% and 19.8% with the epigenetic clock (Marioni et al., 2016). The findings from this study revealed that both TL and DNA methylation were independent predictors of chronological age; but there was only a weak correlation between both the biomarkers of ageing. This shows that the mechanism by which DNA methylation and TL affect ageing differs and this will require further investigations.

1.9 Biomarkers of ageing and frailty

1.9.1 Telomere Biology and frailty

TL has emerged as a novel biomarker of cellular senescence over the years. Research outcomes have suggested that TL/telomere shortening has been associated with ageing, mortality and other age-related diseases which does constitute a part of the frailty syndrome. There are 8 cross sectional studies, with 2 of the studies being longitudinal in nature investigating the role of TL and frailty that has been published thus far (Woo et al., 2008) (Yu et al., 2015) (Collerton et al., 2012) (Saum et al., 2014) (Breitling et al., 2016) (Marzetti et al., 2014) (Brault et al., 2014) (Haapanen et al., 2018). Current evidence suggests that shortened telomeres are reportedly associated with low levels of physical activity (Cherkas et al., 2008). Studies have hypothesized an association between TL and indicators of physical function (e.g. grip strength; a component of frailty) in the elderly but failed to prove their hypothesis (Bekaert et al., 2005; Harris et al., 2006; Mather et al., 2010). However, these studies had relatively small sample sizes and included narrow age ranges. Subsequently, Baylis et.al showed that faster telomere attrition represented by the percentage change in TL over the 10 years follow period in 253 patients was associated with lower grip strength at follow-up (adjusted p= 0.035) (Baylis et al., 2014). The association between telomere attrition and lower grip strength at 10-year-follow-up is accounted for by greater inflammation burden measured in the form of IL-1B, a proinflammatory cytokine. A recent study of

202 patients greater than 60 years of age has shown that there were significant difference between frailty groups and TL whereby a 1 kbp increase in TL reducing the risk of frailty by 0.55 times (CI 95%[0.448-0.697] p<0.01) (Darío et al., 2019).

1.9.2 DNA methylation and frailty

The biological mechanisms that underlie frailty remains poorly understood as frailty itself it a complex process part of ageing. The rate of epigenetic age acceleration has been associated with symptoms of ageing such as frailty (Breitling et al., 2016). There is evidence to suggest that DNA methylation patterns at gene promotor regions are altered in frail individuals, but this relationship is rather a complex one and the importance of it is still largely unclear/unknown.

There have been a few studies that have investigated the relationship between DNA methylation patterns and frailty in older populations. One study that defined frailty status using cluster analysis reported that global DNA methylation was lower in people who were frail compared to non-frail (Bellizzi et al., 2012) but another study that utilized the Fried phenotype for frailty assessment failed to show a similar association (Collerton et al., 2014). Collerton et.al. had examined genome wide DNA methylation levels and individual CpGs sites associated with frailty (TUSC3, TWIST2 , HOXD4, EPHA10, HAND 2) but did find that lower levels of CpG island methylation

were associated with a reduced likelihood of being frail (Collerton et al., 2014). However, averaging methylation levels across 5 age related methylation markers were associated with reduced frailty (Collerton et al., 2014).

One of the largest studies of participants between the age of 50-75years (n=1820 people) investigating DNA methylation patterns in frail individuals revealed that greater epigenetic age acceleration was associated with greater frailty with an increase in frailty index by 0.25% points per year of epigenetic age acceleration (Breitling et al., 2016). Another study that focused on DNA methylation in a narrow-age sample of 70-year olds (n=791) found no difference in methylation patterns between those who were physically frail and not frail as defined by the Fried phenotype (Gale et al., 2018). This study also revealed a significant difference in the proportion of methylation at a single CPG site (cg18314882 in chromosome 8 in the MAF 1 gene) among the frail and non-frail group out of the 485, 512 CpGs measured (Gale et al., 2018). Knowledge of the role of this gene is limited (Khanna, Pradhan and Curran, 2015). Participants who were frail as defined by the Fried Frailty Phenotype had a higher mean extrinsic epigenetic age acceleration; older biological age independent of potential confounding factors (Gale et al., 2018).

Frailty outcomes such as grip strength (Sillanpaä et al., 2018) were associated with epigenetic age acceleration; however, standing balance and chair-rise time were not correlated to accelerated epigenetic ageing (Simpkin, Suderman and Howe, 2017). A study by Marioni et.al from the Lothian Birth Cohort in 1936 (n= 1091) of older individuals ages between 70-76 years showed that

greater DNA methylation acceleration was correlated with lower cognitive score, weaker grip strength and poorer lung function test in humans during later life but not walking speed (Marioni, Shah, McRae, Ritchie, et al., 2015). The authors also demonstrated that the outcomes were similar using both Horvath and Hannum epigenetic clock but the Hannum predictor yielded a weaker cross-sectional association.

These studies emphasize that greater epigenetic age acceleration i.e. biologically older has been associated with greater risk of being physically frail. It is important to recognize frailty as this clinical syndrome is associated with an increased risk of adverse outcomes which include falls, hospitalization, prolonged hospital stay and death. It remains difficult to establish if changes in DNA methylation are a direct causative role of frailty or merely a driving factor behind methylation changes and development of frailty.

1.10 Is there a relationship between ageing, frailty and biomarkers of ageing; TL and DNA methylation in uraemia?

Biological age is much more accurate in reflecting an ageing individuals' overall health wellbeing in comparison to their chronological age. This is largely due to the effect of environmental stress and physiological reserve that differs in the population which accounts for the variation. Both frailty and ageing have some form of commonality. However, growing old in itself is not a prerequisite to becoming frail as discussed previously and not all inter-individual variation can be explained by chronological age alone suggesting an alternative mechanism underlying these changes.

Frailty is a well-recognised clinical syndrome in the dialysis cohort whereby the prevalence of frailty has been reported to be greater than 60% (Bao et al., 2012) (Johansen et al., 2007) in comparison to community dwelling adult older population with a prevalence of frailty up to 11% (Collard et al., 2012). There is growing consensus that frailty does exist as a distinct syndrome which occurs principally in a subset of individuals who are most vulnerable and independently associated with an increased risk of infection, hospitalization, dependency and reduce life expectancy. The concept of frailty is more predictive of an outcome than the chronological age per se even in the dialysis cohort, suggesting that frailty may be a better criterion to identify older persons in need for intervention; a concept that has been echoed throughout various nephrology platforms.

This highlights the gravity and the importance of this issue as we are yet to know the exact aetiology of frailty in the dialysis cohort and the underlying basis for differences between individuals. Factors that contribute to frailty in dialysis cohorts includes chronic inflammation, accumulation of uraemic toxins, metabolic acidosis, sarcopenia and protein energy wasting. There is an overlap between frailty and protein energy wasting. The relationship between these 2 components are often dependent and affect each other. (See Figure 8)

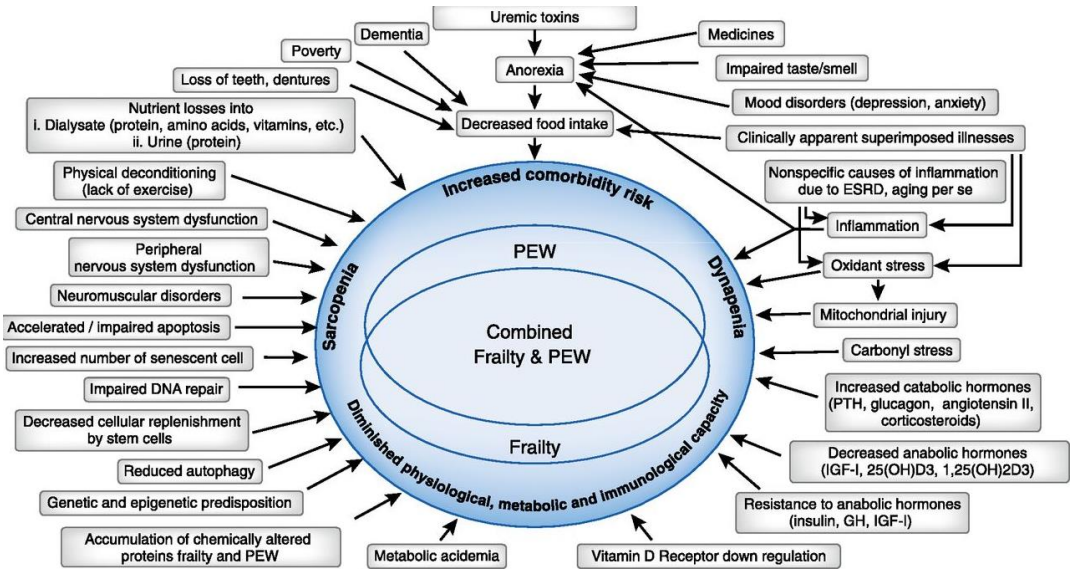

Figure 8: Causes of frailty and protein energy wasting observed in dialysis patients. Reproduced with permission (Kim, Kalantar-Zadeh and Kopple, 2013).

Dialysis patients are affected by the factors outlined in figure 8. These are associated with and contribute to premature ageing. TL is also affected by similar factors commonly found in uraemia; inflammation and oxidative stress.

Therefore, TL is not only an ideal biochronometer representing biological age but could hypothetically be a biomarker to assess frailty and ageing in ESKD.

The area that has yet to be explored in the dialysis cohort is the role of epigenetic clock in accelerated ageing and frailty, as epigenetic mechanisms have been suggested as a factor/cause of frailty. Epigenetic markers in the form of DNA methylation status will be remodelled in response to the lifetime exposure to different environment stresses. Epigenetic factors; in particular DNA methylation vary significantly across one's life time and in populations of similar age (Rakyan et al., 2010). There is also a genetic basis for frailty as shown by Kim et.al revealing that the pattern of ageing differed among offspring of long-lived versus short lived parents (Kim and Jazwinski, 2015). DNAm status may provide the clue and answer to the phenotypic changes observed in the dialysis cohort as both metabolic changes and co-existing inflammation has a greater effect on epigenetic mechanisms. Perhaps the most exciting feature is that the epigenetic changes are reversible, raising the prospect that DNAm status estimates might thus be useful for identifying or validating anti-ageing interventions in this cohort.

Identification of these robust associations between DNAm and TL as a marker of biological ageing may provide potential diagnostic and therapeutic avenues in managing frailty risk in uraemia. These biomarkers may not replace existing clinical biomarkers but may aid in patient care especially in cohort of patients whereby routine clinical biomarkers may not provide an accurate finding due to the nature of the disease. The biomarkers of ageing can help determine a

diagnosis of frailty and subsequently help prompt intervention on known, recognised and modifiable factors that could reverse frailty. Reversing frailty on time could certainly improve quality of life, mortality outcomes and reduce hospitalisations.

Research in DNA methylation and TL in the context of uraemia is still in its early days as most research outcomes and studies have been conducted in non-dialysis cohorts. However, studies in the non- dialysis cohorts have paved the way and generated the hypothesis for this PhD. One of the aims of the thesis was to assess a functional/phenotypic consequence in the form of frailty assessment with DNA methylation and TL changes. It is not known if the changes in TL and methylation correlate to changes in expression are causative and functionally important in ageing phenotype, but further studies are required to investigate these research questions.

CHAPTER 2: AIMS

2.1 An overview of Rationale, Aims and General Hypothesis of the research

Chronological age does not reflect the physical state of an organism and does not occur at the same rate in individuals. There is a growing body of evidence to suggest that patients with end-stage renal disease develop accelerated ageing, the mechanisms of which are not fully elucidated. For example, ESKD patients develop cardiovascular events at a much earlier age than their normal-control counterparts when matched for age and gender. ESKD patients also appear older than their age as evidenced by sarcopenia, skin thinning, bone loss, cognitive dysfunction, frailty etc. It is crucial to recognise that premature ageing among ESKD population is reflected by a decline in physical function, frailty phenotype and overall well-being which is a major health agenda. The frailty phenotype which in recent years has gained increasing awareness among health professionals caring for patients on dialysis due to the association between frailty with adverse outcome has highlighted the importance of this area in dialysis and transplantation. Nephrologists are recognising the impact of frailty on the dialysis patients and implications in future of decision making e.g. renal transplantation in borderline but younger dialysis patients, initiating dialysis in frail patients and escalation of care of these vulnerable dialysis patients. However, there is no agreed consensus on the best frailty assessment approach and there remains to be insufficient evidence to recommend a specific screening tool or even which tool is better.

There is a lack of research investigating for a reliable objective marker of accelerated ageing which could also be a potential biomarker that could reflect one's frailty status in ESKD which forms the initial aim of this research and thesis.

Telomeres are a biologically plausible candidate to explore ageing in this cohort because of its essential role in genomic stability and normal cellular function with both in vivo and in vitro studies suggesting that TL reflects levels of oxidative stress and cellular senescence; both factors are hallmarks of ageing in dialysis patients. The other aspect of this study is the role of DNAm, an epigenetic marker highly correlated with chronological age as another potential biomarker of ageing. This PhD project aims to look into TL and DNAm status in patients with end stage renal disease with the aim to identify if this marker of biological ageing can help identify individuals at greatest risk of adverse outcome in the form of frailty status.

This area is an important one as it provides important opportunities for targeting prevention and intervention. Investigating how TL and the estimated epigenetic age differs across a group of individuals of the same chronological age could help determine the impact of endogenous or exogenous stress factors on biological ageing. Based on my current literature review it is evident that there a still many gaps in the literature surrounding the use of TL and DNA methylation as a biomarker of ageing and frailty in patients with ESKD as well as a biomarker of cumulative oxidative stress and inflammation.

The principle aim of this thesis is to further explore and investigate the relationship between ESKD, TL, DNAm markers and frailty.

2.1.1 Summary of hypothesis

We postulate that patients with ESKD would have shorter TL suggestive of premature ageing in comparison to a healthy cohort. We were also keen to analyse TL shortening in patients with ESKD longitudinally to determine TL attrition rate and investigate any correlation between TL; a biomarker of ageing and oxidative stress with frailty phenotype in the dialysis cohort. We hypothesize that dialysis patients will have significant telomere attrition over a year. DNAm was chosen to assess if DNAm markers could be surrogate for TL. This work will be a hypothesis-generating study which may lead to randomised control trials investigating the role of TL and DNAm status as ageing parameters in ESKD.

2.2 General Aim of the study

The general aim of the study is to investigate the role of TL, DNA methylation and frailty status as biomarkers of ageing in ESKD patients.

2.2.1 Aim of TL analysis in the study cohort

1) To compare TL measurement between a healthy cohort as control with patients on dialysis
2) To explore if there are any difference between TL in HD and PD patients
3) To determine if TL is associated with the development of frailty in the dialysis cohort

2.2.2 Aim of DNA methylation analysis in the study cohort

1) To determine if DNA methylation markers are associated with the development of frailty in the dialysis cohort
2) To investigate the association between TL and DNA methylation markers

2.2.3 Aim of telomere attrition and frailty changes over a year

1) To investigate telomere attrition in the dialysis cohort over a 12-month period
2) To investigate changes in frailty status at baseline and over a year

CHAPTER 3: CLINICAL METHODS

3.1 Introduction to study design

The clinical aspects of the study design and execution are elaborated in the section below.

3.1.1 Study design and patient recruitment

This research was based on an observational prospective cohort study that was conducted with the current revision from the declaration of Helsinki and carried out in accordance with Good Clinical Practice as set down in ICH E6. Ethical approval was obtained from Bromley National Research Ethics Service committee. (IRAS Project ID 167266, REC reference 15/LO/1073) (Refer to Appendix A for Study Protocol Version 4.0 and Appendix B for Full set of project data).

Patients were recruited from Bart's Health NHS dialysis units and renal outpatient clinics from December 2015 until July 2018 as per the inclusion and exclusion criteria outlined below. No inpatients were recruited for the study due concerns of the effect of an acute illness and inflammation has on telomere length by upregulations of oxidative stress. The study also excluded any patients with known malignancy (active disease or in remission) due to the associations between gastrointestinal cancer (Zhu *et al.*, 2016) and haematological malignancy (Zhu *et al.*, 2016) on TL.

Inclusion criteria

- Any patient with ESKD established on HD or peritoneal dialysis treatment in the outpatient setting
- Any potential kidney donor

Exclusion criteria

- Any inclusion criteria not met
- Lack of capacity
- Known history of malignancy at point of recruitment
- Any patient with known history blood borne viruses
- Felt to have a life expectancy of less than 6 months
- Less than 18 years of age
- Acute infection and/or in-patients having treatment

Suitable patients were approached with a patient information leaflet prior to obtaining informed written consent to participate in the study (Refer to Appendix C for patient information sheet version 2, Appendix D for consent form version 2 and Appendix E for GP letter version 1). Consented study patients were followed up for a period of 1 year after recruitment. Any refusers or dropouts were clearly documented.

3.2 Phenotypic investigation and assessment

Basic demographic characteristics at baseline (age, gender, sex, ethnicity, past medical history, cause of ESKD, Charlson comorbidity index, dialysis vintage, dialysis access, dialysis adequacy, smoking history, medication history, height and weight, full blood count, white cell blood count, c-reactive protein) and 1 year follow up data (weight, hospitalisations, mortality and survival outcomes) were extracted from electronic health records, through questionnaires developed for the purpose of this study (Refer to Appendix F for Frailty Measurement Part 1 and Overall Progress) and direct questioning whenever possible.

The Charlson comorbidity index of the study recruits are determined based on patients' age and 16 conditions with its designated score as seen in Table 1. The more points accrued by the patient, the more likely the predicted adverse outcome. Patients were than grouped into four different categories based on the sum of the final points; score 0 (none), score 1-2 (low), score 3-4 (moderate) and score ≥5 (severe).

Score	Condition
1	Myocardial infarction (history, not ECG changes only)
	Congestive heart failure
	Peripheral vascular disease (including aortic aneurysm ≥6cm)
	Cerebrovascular disease: CVA with mild or no residua or TIA
	Dementia
	Chronic pulmonary disease
	Connective tissue disease
	Peptic ulcer disease
	Mild liver disease (without portal hypertension, includes chronic hepatitis)
	Diabetes without end-organ damage (excludes diet controlled alone)
2	Hemiplegia
	Moderate or severe renal disease
	Diabetes with end organ damage (retinopathy, neuropathy, nephropathy, or brittle diabetes)
	Tumour without metastases (exclude if >5y from diagnosis)
	Leukaemia (acute or chronic)
	Lymphoma
3	Moderate or severe liver disease
6	Metastatic solid tumour
	HIV positive with AIDS defining illness

Table 1: Charlson Comorbidity Index (CCI) Scoring System. For each decade of >40 years, a score of 1 is added to the scores from the table above to obtain a final score.

Dialysis vintage was defined as total time (in weeks) on dialysis up to the point of recruitment for the study. Smoking habit were recorded as current smoker, former smokers and non-smoker. Development of cardiovascular disease during the follow up period was defined as clinical history or signs of ischaemic cardiac disease and/or presence of peripheral vascular disease and/or cerebrovascular disease. Whilst cardiovascular mortality was defined as death resulting from coronary heart disease, sudden death, stroke or complicated peripheral vascular disease. The cause of death was registered by a health professional blind to both clinical and biochemical data of the patients as well as the objective of the study. Survival was determined from the day of sample collection.

3.3 Frailty assessment

The presence of frailty was evaluated in accordance to the definition by Fried et.al. The assessment is based on 5 domains (see Table 2); weakness, poor endurance/energy, physical inactivity, slowness and shrinking.

Components of frailty	Description of assessment
Weakness	Meets criteria for frailty if grip strength on 3 average trials measured with an analogue hand grip dynamometer (Takei, UK) of the dominant or non-fistula arm as below: Men — Women ≤29kg for BMI ≤24 — ≤17kg for BMI ≤23 ≤30kg for BMI 24.1-26 — ≤17.3kg for BMI 23.1-26 ≤30kg for BMI 26.1-28 — ≤18kg for BMI 26.1-29 ≤32kg for BMI >28 — ≤21kg for BMI > 29
Poor endurance & energy	Exhaustion based on 2 questions from the CES-D Depression scale: 1) I felt that everything I did was an effort 2) I could not get going How often in the past week did you feel this way? Patients who felt either way for ≥3 days in the past week were considered frail.
Slowness	Walking time of 15 feet (4.57m) adjusting to gender and height Considered frail if normal walking pace is: Men — Women ≥7 seconds for height ≤173cm — ≥7 seconds for height ≤ 159cm ≥6 seconds for height >173cm — ≥6 seconds for height >159cm
Low physical activity	Based on short version of Minnesota Leisure-Time Physical Activity Questionnaire. (See Appendix G for Frailty Measurement Part 2: Minnesota Leisure Time Physical Activity Questionnaire). Frail if Male <383 kcals/week and Female <270 kcals/week

Shrinking	Considered frail if ≥10 pounds (4.5kg) of unintentionally weight loss (i.e. not due to dieting or exercise) in prior year. At follow up, weight loss was calculated as: (Weight in the previous year-current weight)/ (Weight in the previous year) = K. If K≥0.05 with unintentional weight loss of at least 5% of previous year's body weight, then considered frail for weight loss.

Table 2: Fried Frailty Phenotype Scoring Criteria. (Refer to Appendix F: Frailty Measurement Part 1 and Overall Progress and Appendix G: Frailty Measurement Part 2: Minnesota Leisure-Time Physical Activity Questionnaire)

Frailty assessment was completed at the time of or within 4 weeks of blood sample collection at recruitment (time point 0) and at 1 year follow up (time point 1). Patients on HD had frailty assessments completed prior to dialysis treatment during a midweek session. We chose to assess all patients pre dialysis for 2 reasons: firstly, patients were more available before dialysis while waiting to start and not wanting to wait around after dialysis particularly those on transport. Secondly to reduce any effects of the acute dialysis treatment on the frailty assessments, for example many patients may intermittently feel 'washed out' immediately post dialysis. Studies have demonstrated that hand grip strength and walking speed is affected by dialysis treatment due to post dialysis associated fatigue (Jhamb *et al.*, 2008) (Pinto *et al.*, 2015).

All remaining patients were assessed at outpatient clinics. All the frailty components were recorded using the convention that '0' indicates the absence of a deficit, and '1' indicates the (full) presence of a deficit. On the basis of the assessment, study recruits were given a final score and classified into one of the three frailty stages; non-frail (score 0), pre-frail (score 1-2) and frail (score 3-5).

3.4 Patient recruitment for the study

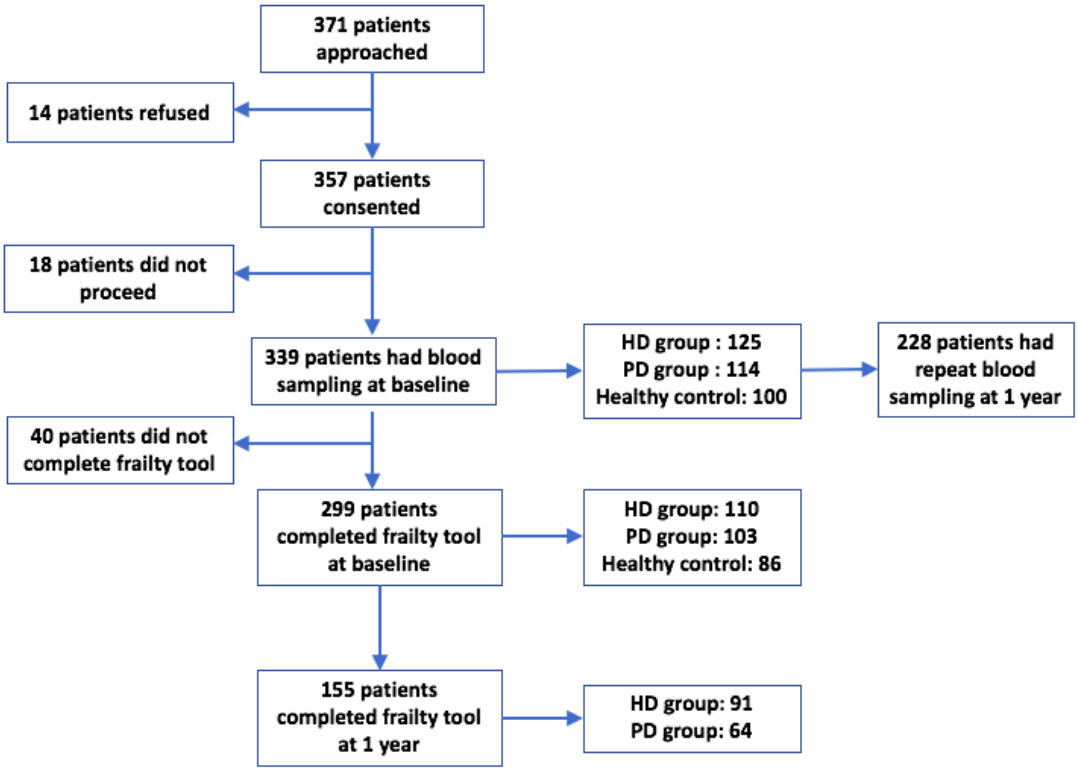

Figure 9: Flow chart on patient recruitment during study period of 1 year

Patients were recruited from Barts Health NHS Trust between the period of December 2015 to May 2018 from the dialysis units and outpatient clinics. A total of 371 patients were approached with patient information leaflets and 357 had provided written consent for the study. Unfortunately, 18 patients did not proceed with the study and the remaining 339 patients who consented had their blood samples taken as per protocol. Only 299 patients had Frailty Assessments completed in full and 40 patients who had already provided blood samples subsequently did not complete the questionnaires in the study protocol.

Reasons for patients not proceeding with the study or not completing Frailty Assessments are as below:

a) having changed their mind subsequently
b) moved to another trust
c) absent on the day of their scheduled blood test and frailty assessment
d) unexpected hospitalisation

Only study participants from the dialysis arm were followed up for a 12-month period. Frailty assessments were completed by 155 patients out of the 299 patients who had also completed baseline assessments for frailty. Blood sampling at 12-month follow up period was taken from 228 patients.

Reasons for patients not having their blood sampling or completing their frailty assessments at 12-month follow up are similar to reasons as to why patients had not completed baseline assessments.

3.5 Statistical Analysis

Each continuous variable was tested for normality using the Shapiro Wilks test. The data for continuous variables with normal distribution were expressed in the form of mean ± standard deviation (SD). Any other continuous variables that were not normally distributed was reported in the form of median ± interquartile range (IQR). All categorical variables were presented in percentages or ratios.

Categorical variables were compared using Chi-Square, Fishers exact and McNemars test. Numerical variables were compared with various T test, ANOVA, Kruskal Wallis, Mann Whitney U-test and Wilcoxon test. Univariate and multivariate analysis was used to evaluate differences between variables and simultaneously adjusting for confounding variables. The association of variables were tested using Pearson's or Spearman's correlation based on the data distribution.

All the statistical analysis was performed using the IBM Statistics Package for Social Sciences (SPSS) version 25 for MAC and R version 3.0.1. P values < 0.05 (two sided) were regarded as significant for the purpose of this thesis.

CHAPTER 4: LABORATORY METHODS ON TELOMERE LENGTH

4.1 Blood sample collection and buffy coat isolation

Blood samples were collected in a 6ml EDTA vacutainer tube for all study recruits. For patients undergoing HD, blood sampling was done before the start of dialysis treatment and during a midweek session via their existing access whilst all the other study recruits including peritoneal dialysis patients were bled from their peripheral vein when attending clinic. All samples were centrifuged for 10 mins at 2500G at room temperature to isolate the buffy coat layer within 30minutes of sample collection. Buffy coat layer was cautiously transferred into polypropylene tubes of 2ml volume for storage. DNA extraction was performed immediately and remaining buffy coat were stored at -80°c for future use.

4.2 DNA Extraction Materials and Protocol

Genomic DNA was extracted from isolated buffy coat using an automated DNA extraction machine (Qiagen Bio Robot EZ1) with the aid of a prefilled reagent cartridge (EZ1 DNA Blood 350µl kit from Qiagen, Cat No./ID: 951054) according to manufacturer's protocol. The kit contained all the required reagents and lab ware for rapid, automated purification of genomic DNA from 300µl of buffy coat using a

magnetic particle technology. The final elution volume of purified genomic DNA was 200μl.

4.3 DNA Quantification and Quality Control

Extracted genomic DNA was assessed for purity and quantified with a Thermo Scientific Nanodrop^{TM} 2000/2000c spectrophotometer. Calibration of spectrophotometer was performed first using DEPC- Treated Water (nuclease free) prior to measuring 1μl genomic DNA in duplicates. The spectrophotometer provides the quantity of the measured DNA in ng/μl and quality in the form of the 260/280 ratio.

Nucleic acid has a peak absorbance in the ultraviolet range rate at 260nm. Whilst protein has a peak absorbance at 280nm. A ratio of absorbance at 260nm and 280nm of ~1.8 is generally accepted as 'pure' for DNA. Any samples with a 260/280 ratio of less than 1.8 was excluded from further analysis due to probable protein contamination and repeat DNA extraction is done for these samples. The repeat DNA is re-assessed for quality and quantity with a spectrophotometer and the sample was excluded for further analysis if the 260/280 ratio remained outside the defined range for the study.

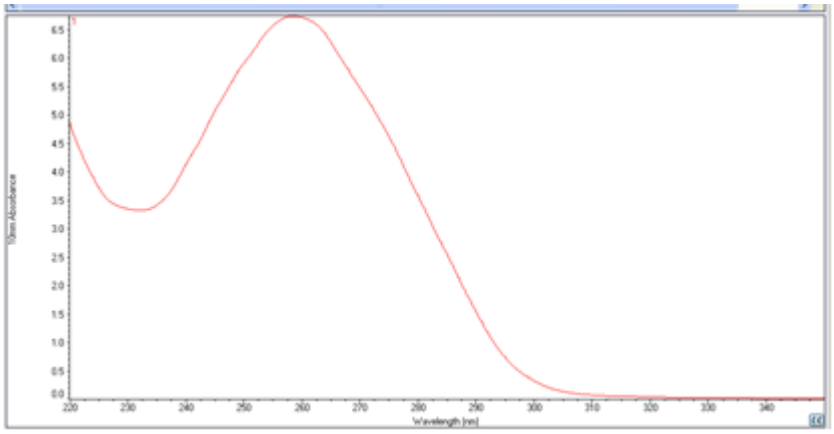

Figure 10: Typical pattern of nucleic acid spectrum seen in study samples included for further analysis

4.4 DNA integrity using gel electrophoresis

All genomic DNA integrity was assessed by gel electrophoresis. Approximately 10µl of genomic DNA was used for this purpose. The samples were run on a 0.7% Agarose gel. The gel is made by dissolving 1.4g of Agarose MP powder (Sigma-Aldrich, Cat. No 11388983001) in 200mls of Tris-acetate-EDTA (TAE) buffer 1x (Premix TAE Buffer 10x, Sigma-Aldrich, Cat. No. 11666690001) by heating in a microwave until boiling. The solution is poured into gel casts with a comb in-situ and any bubbles were removed with a pipette tip. The gel was subsequently transferred to a gel tank once it had set and the comb removed.

Each sample was mixed with 5µl of tracking dye (Sigma, Cat No: G2526) to make up a total volume of 15µl before being loaded into the wells of the gel. The tracking dye

adds density to the final mixture to facilitate sample loading and allows visualisation of the sample migration. A 10µl DNA marker mixed with 5µl of tracking dye was loaded at extreme ends of the gel which is than run at 90V. Once the marker has migrated ¾ of the gel from the loading position, the gel runner is switched off and the gel is transferred into a container filled with approximately 200mls of distilled water and 50µl of Gel Red Nucleic Acid Gel Stain (Cambridge Bioscience, Cat No. BT41003). The gel is left on a slow shaker for 60 minutes. Following this period, the coloured solution was discarded, and the gel was visualised under an ultraviolet (UV) trans illuminator to establish DNA integrity. Intact DNA will appear as a band under UV light with no smear below the band. If there is a smear after the band then this suggests that the DNA is degraded and excluded from further analysis.

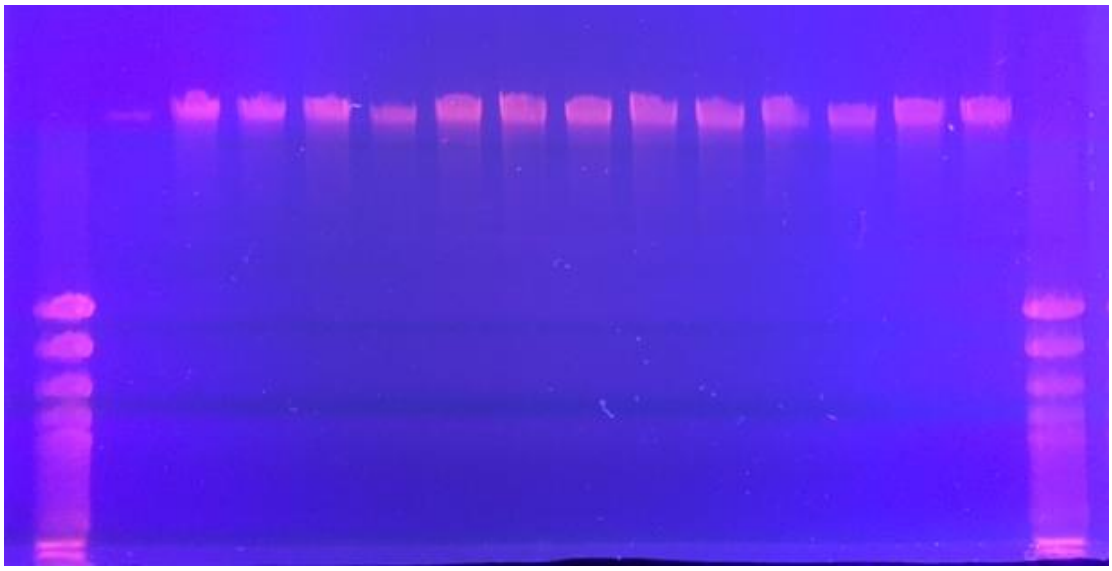

Figure 11: Agarose gel showing intact DNA with no evidence of degradation

4.5 Standardisation of DNA

Stock samples of genomic DNA of 100µl was stored in -20° for long term use. The remaining 100 µl of stock DNA was than standardised to a concentration of 10ng/µL and aliquoted into a 96 well deep ABgene PCR plate and stored at 4°c for short term used.

4.6 Rationale for chosen methodology for TL analysis

TL is measured from peripheral blood leucocytes and is often used as a proxy for TL in other tissues (Daniali *et al.*, 2013) There is generally a good correlation in TL between different tissue compartments of an individual and blood is an easily accessible tissue. Different methods have been utilised to measure TL which include terminal restriction fragment length analysis using Southern Blotting, polymerase chain reaction-based techniques and fluorescent in-situ hybridisation (FISH) technique. The current gold standard for TL measurement is Southern Blotting. Unfortunately, this technique is rather laborious. It takes up to 3-5 days and a large amount of DNA (3.0ug per sample) is required (Kimura *et al.*, 2010). The chosen approach to answer the research question is to use RT-PCR to determine the relative TL. This method is quick, inexpensive and will allow analysis of larger samples (Cawthon, 2002).

4.7 Polymerase chain reaction and its principles

Polymerase chain reaction or better known as PCR is based on the ability and nature of the DNA to self-replicate. The method also relies on the use of short primers to initiate synthesis of a target sequence by a DNA polymerase.

4.7.1 The basic steps involved in PCR

a) Hot start step

Both telomere and single copy gene run is set at 95 degrees for 10 minutes initially. This step is required to activate the Hot Start Taqman DNA polymerase, which prevents amplifications of primer dimers and other non-specific products in the reaction. The fluctuating temperature does not denature the DNA polymerase used in this assay during the PCR.

b) Denaturation step

The double stranded DNA is first heated up to a high temperature for a brief period of time in order to denature the double strand of the DNA into single strand templates required for the next step.

c) Annealing step

During this step, the primer anneals to the complementary bases of the single strand templates of the DNA.

d) Extension step

New complimentary strands of DNA are synthesised at the 5’ end of each primer as initiated by the polymerase.

The process outlined above accounts for 1 cycle of the PCR and this process is repeated multiple times. Each time the cycle repeats itself, this will result in the synthesis of a new piece of double stranded DNA. Therefore, only a small initial amount of DNA is required to subsequently produce a large amount of a specific sequence of interest. Theoretically, each cycle of a PCR would lead to the doubling of a target sequence resulting in an exponential increase of the PCR product. However, in practice the increase in PCR product will depend on the amount of the reaction component remaining in excess for further PCR cycles. After a number of cycles, the rate of amplification will finally reach a plateau phase due to a) saturation of the amplified target sequence b) eventual failure of the DNA polymerase and c) the consumption of dNTPs (triphosphate deoxyribonucleotides). Once a plateau phase has been achieved, subsequent cycles will eventually lead to the formation of primer-dimers and non-specific products.

There are 2 types of PCR widely used. An end point PCR is used to detect a specific sequence and the PCR product is determined by gel electrophoresis or high-resolution melting. Whilst real time PCR is used to quantify a known target sequence; as in this

study the target sequence of interest was TL. In real time quantitative PCR, the amplification of the PCR product is measured by the fluorescence emitted from the chosen probe in each cycle.

4.8 Real time quantitative PCR protocol to measure TL

The protocol to measure TL for this study was based on the methodology developed by Richard Cawthon with an improvised set of primers using real time PCR (Cawthon, 2002). Longer telomeres will have more potential primer annealing sites and hence an increase in the fluorescence and reduction in the number of cycles needed to reach a given threshold in comparison to a shorter telomere. This concept allowed the ability for researchers to measure TL due to the simplicity of the technique and amenability to enable rapid high throughput processing of samples at a large scale. All PCRs were performed on the Rotor Gene 6000 (Corbett Research), a thermal cycler.

4.8.1 Primer design

The primer sets for telomere were Tel 1b and Tel 2b and 36B4 for single copy gene (SCG). The telomere primers were HPLC purified to avoid primer-dimer formation from primers that are not full length which are found in lower purification synthesis. Primers for telomere run; Tel 1b and Tel2b are both diluted to a final concentration of 300nM. Whilst primers for SCG run; 36B4F and 36B4R were diluted to a final concentration of 300nM and 500nM.

Telomere PCR Primers and Thermal Profile

Telomere Forward Primer (Tel 1b): CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT 5'-3'			
Telomere Reverse Primer (Tel 2b): GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT 3'-5'			
Thermal Profile	Number of cycles	Temperature	Duration
Activation	1	95°c	10 min
Amplification	20	95°c	15 sec
Cooling	1	58°c	1 min

Table 3: The primer sequences and thermal profiles for telomere PCR amplifications

36B4 PCR Primers and Thermal Profile

36B4 Forward Primer: 5'-3': CAGCAAGTGGGAAGGTGTAATCC 36B4 Reverse Primer: 3'-5': CCCATTCTATCATCAACGGGTACAA			
Thermal Profile	Number of cycles	Temperature	Duration
Activation	1	95°c	10 min
Amplification	30	95°c	15 sec
Cooling	1	58°c	1 min

Table 4: The primer sequences and thermal profile for 36B4 PCR amplifications

4.8.2 Preparation of reaction mix

The reaction mix composition is made up of SensiMix ^{TM} SYBR No-ROX Kit reagent (Bioline, Cat.No. QT650-05), primers at the concentration described in Section 4.8.1 and genomic DNA at 10ng/µl. Master mixes for both runs are made up at the same time to ensure the same enzyme mix is used. Each run had a negative template as control (NT) and a calibrator sample (K562 Genomic DNA, Promega). The final reaction volume was 25µl. Each sample was plated in duplicates.

4.9 PCR run and results from real time quantitative PCR

Both telomere (T) and 36B4 (S) runs were performed separately as per the cycling conditions described in Table 3 and 4. The T run is performed for the samples first followed immediately by the S run to keep all conditions uniformed. The section below outlines the results of the RT-qPCR of TL analysis performed in this study and outlines the overall quality of the RT-qPCR runs performed for TL assay.

4.9.1 Standard Curve

The aim of a standard curve in RT-qPCR is to provide relative concentration of samples, demonstrate efficiency and accuracy of the assay. A standard curve was not included in each plate/run to allow higher throughput as well as to eliminate

inaccuracies from dilution error when preparing standard curve samples. However, a standard curve was performed prior to preparing TL RT-qPCR assay to demonstrate primer and master mix efficiencies.

In this protocol, the standard curve was used solely for the purpose of ensuring primer and overall PCR run efficiency. The standard curve generated for TL assay protocol consisted of 8 different concentrations of DNA from a pooled sample at 2-fold dilutions from 100ng/µl to 0.78ng/µl.

Figure 12 and 13 demonstrate a one cycle difference for each standard curve which represents a dilution factor of 0.5 revealing accuracy of the dilution process performed subsequently demonstrated in Figure 14 and 15. These results highlight the linear correlation between the serial dilution performed and the amount of target sequence copies measured, which indicates the accuracy of the quantification process in RT-qPCR. Demonstrating this step is an important quality control measure prior to running samples of interest with the RT-qPCR protocol.

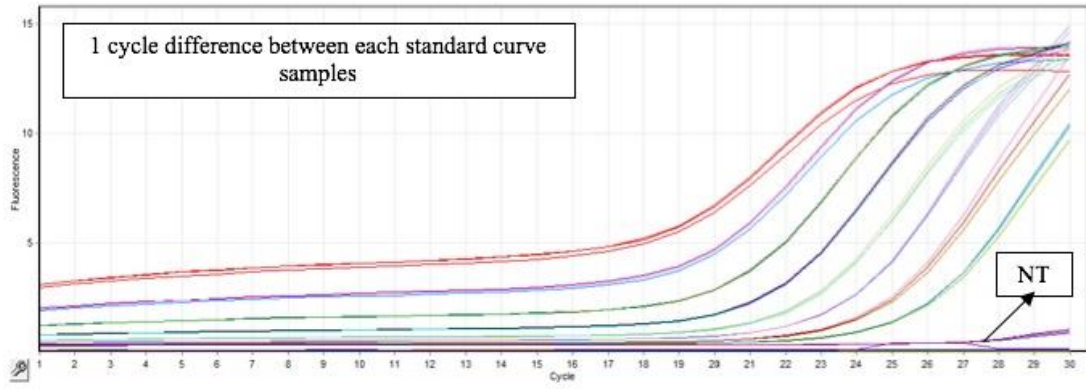

Figure 12: Standard curve appearance for SCG RT-qPCR

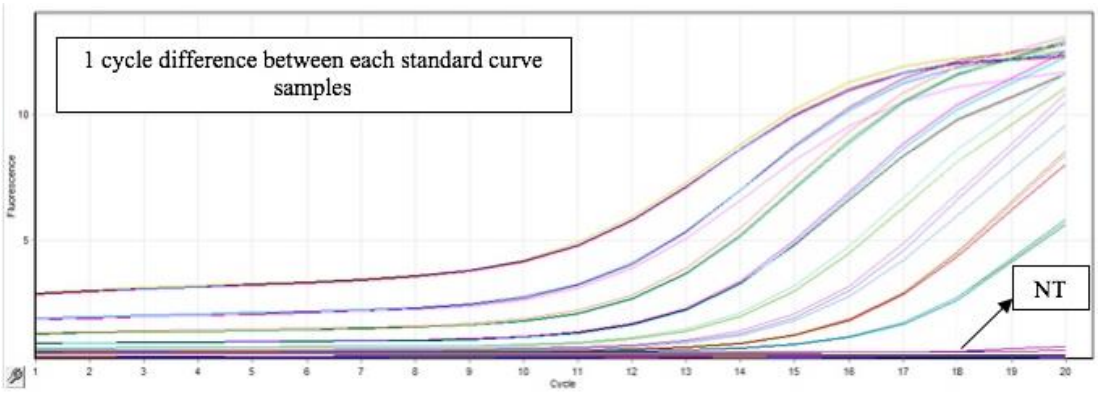

Figure 13: Standard curve appearance for Tel RT-qPCR

Efficiency (E) is calculated by the slope (M) of the curve with the formula $E=10^{-1/M}$. Slope (M) of a reaction is used to determine the exponential amplification and efficiency of the standard curve reaction. Optimal values for M, amplification and reaction efficiencies are -3.322, 2 or 1 respectively.

Under ideal conditions the amount of template is doubled in each PCR cycle which gives an efficiency of 100%. Lower efficiencies suggest problems from the primer design and master mix performance. Efficiencies above 100% would indicate the probability of primer-dimer formations. Generally, efficiencies between 90% and 110% are acceptable as seen in the standard curve generated for the TL assay in this study.

Efficiencies were calculated using the Rotor-Gene Q Series Software. For both the assays, the linearity observed over the chosen range of DNA concentration was > R^2= 0.99 as seen in Figure 14 and 15.

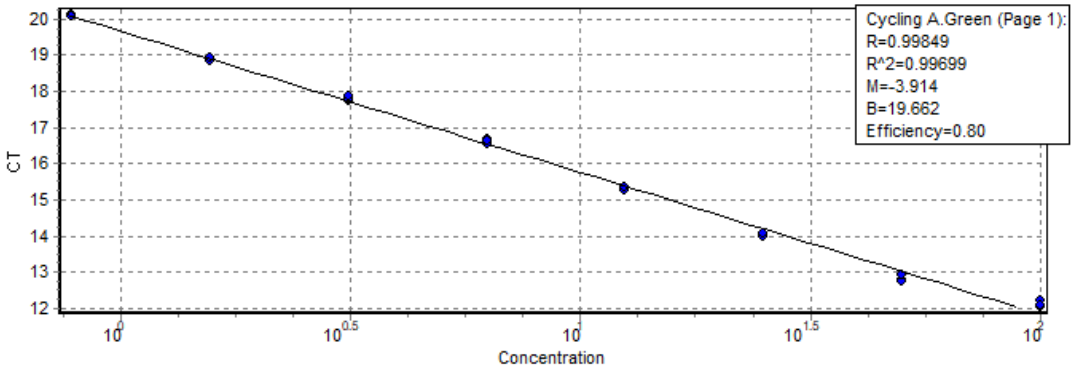

Figure 14: SCG standard curve assay precision and efficiency generated by rotor gene analysis software

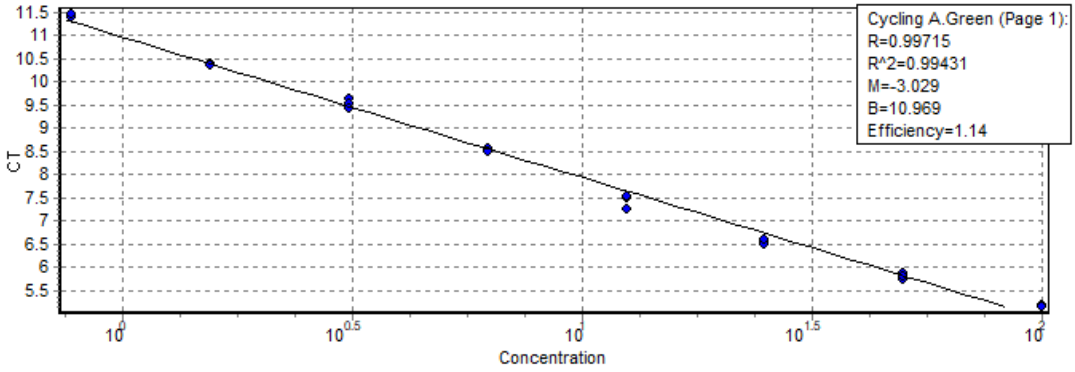

Figure 15: Tel standard curve assay precision and efficiency generated by rotor gene analysis software

4.9.2 TL RT-qPCR assay

The figures below show an example of the RT-qPCR assay performed to measure TL which consists of 48 samples, the calibrator and NT. Each sample was measured in duplicate. The average amplification of each sample and run should be > 1.6. Samples and runs that failed to achieve these amplifications were repeated. The overall average amplification of Tel run was 1.83 ± 0.01. The overall average amplification of SCG run was 1.74 ± 0.01. Figure 16 and 17 shows that there was no amplification seen in the NTC which reflects no evidence of contamination in the run.

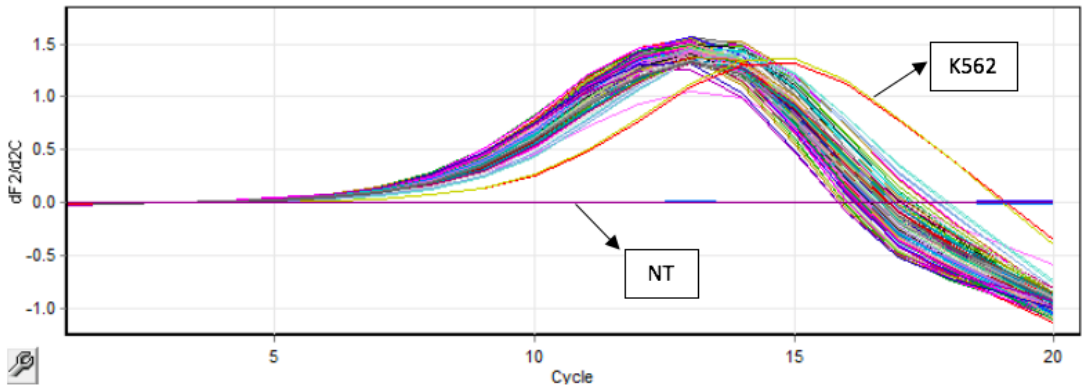

Figure 16: RT-qPCR of Tel run with study samples

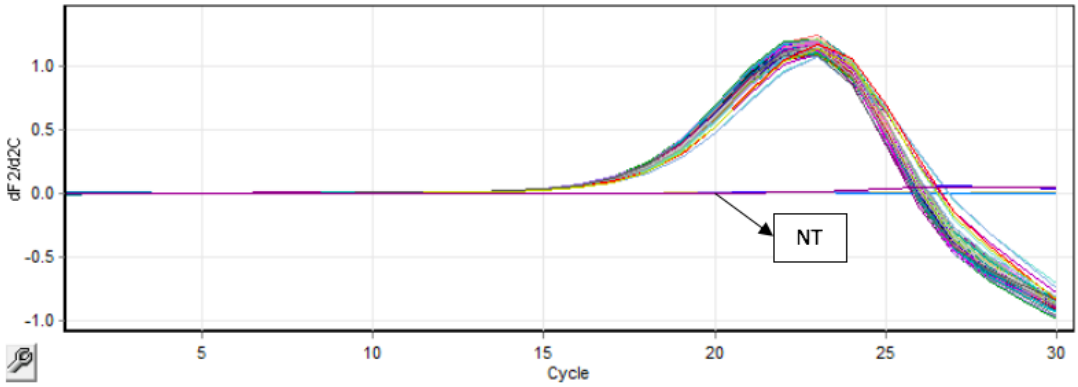

Figure 17: RT-qPCR of SCG run with study samples

4.9.3 Melt Curve

The melting profile was interrogated once the PCR run is complete. Determining the melting point is an important step to validate the amplicon signals. It also helps identify if any non -specific products have been detected during amplification.

The fluorescence in the melt curve analysis is initially high because the sample starts as a dsDNA. The peak point in the melt curve represents the melting temperature (T_m) of telomere DNA fragment. The fluorescence will continue to decrease as the temperature continues to rise as the dsDNA dissociate into single strands. As shown in Figure 18 and 19, the melting curve has only one single peak at ~ 82.5^0c which is the telomere PCR product. The NTCs appear to have generated a non-significant amount of primer dimer product or PCR contamination as seen in Figure 18.

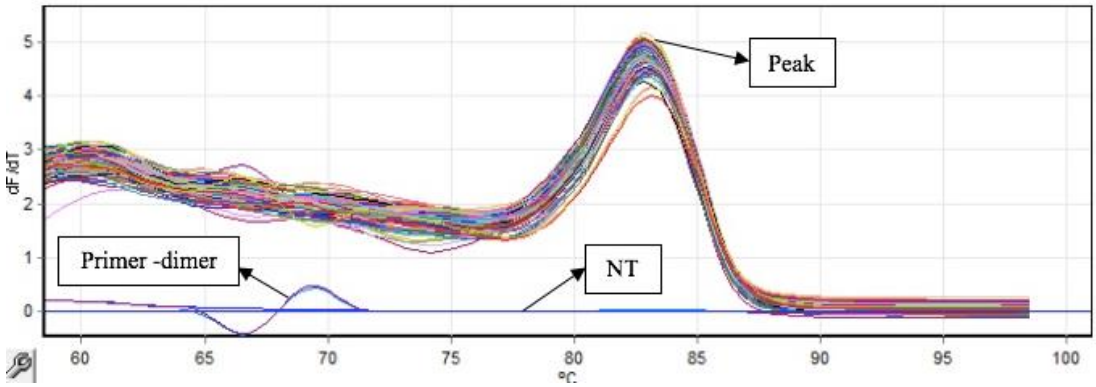

Figure 18: Tel run melt curve

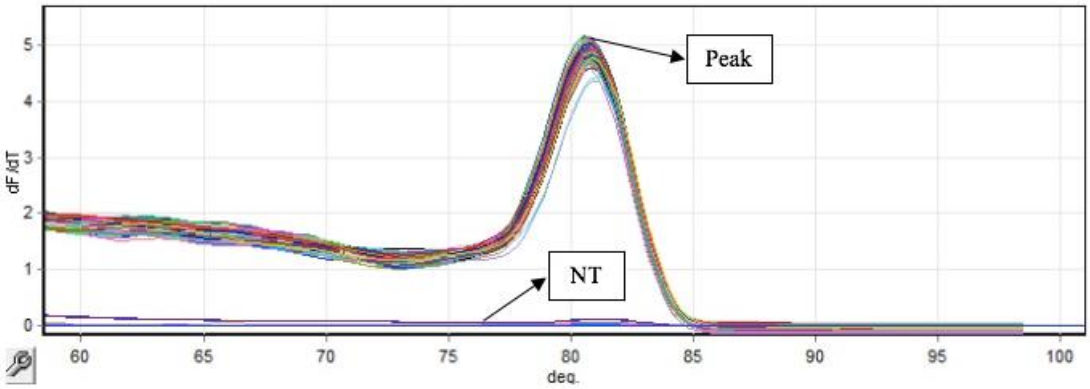

Figure 19: SCG run melt curve

4.9.4 TL Analysis from RT qPCR

There are several ways to analyse the raw data obtained when determining relative TL from real time qPCR. These analysis methods include the standard curve concept, $\Delta\Delta C_T$ analysis, Pfaffl method and comparative quantification (Lin *et al.*, 2019)

The chosen method of analysis for the purpose of this research project was based on the comparative quantification method. This method is well established, widely used and published (McCurdy, McGrath and Mackay-Sim, 2008). The principle of this type of analysis is based on measuring the relative expression/concentration of the sample of interest to a control sample/calibrator without the need to rely on a standard curve. The analysis is performed with the aid of the Qiagen Rotorgene Comparative quantification software.

This method calculates the efficiency for each sample of interest based on the change of fluorescence and mean efficiency across the run rather than taking values from a set based on standard curves. This increases the accuracy of the analysis compared to the other methods.

The software plots the second derivative of the amplification plot whereby the peak of this curve would be the maximum exponential growth of the product. The point 80% back from the peak is defined as the take-off point and the reaction efficiency is calculated from the slope between take-off and the point where maximum amplification stops, also known as the start of the exponential phase. This measure is

less subjective as it avoids the investigator having to set the threshold value/line or depending on the auto threshold mode for each run to give the C_T results in other analysis methods. The threshold value/line is best placed in the exponential amplification across all of the amplification plots for each run which can be variable and contribute to inconsistent results.

The software calculates the mean amplification efficiency (MAE) of all samples in the run. The concentration of each sample relative to the calibrator (reference) sample is calculated using the equation:

$$\text{Sample concentration} = \text{MAE}^{(\text{Calibrator take-off} - \text{sample take-off})}$$

This is done for both the T run and S run for each sample duplicate and TL is expressed as T relative conc. / S relative conc. (T/S). This method takes both the differences in amplification efficiency between samples and between runs into account. All data is relative to the K562 calibrator sample, minimising run-to-run variation.

4.9.5 Quality control and reproducibility of the method

Each sample was analysed in duplicates to assess for intra-assay variability. Any sample whereby the duplicate value in the RT-qPCR run had a cycle difference for the take-off value of >0.2 was excluded from the run and repeated later. Any sample or run with an average amplification of <1.6 was excluded and repeated again.

The TL assay for this study used genomic DNA from K-562 cell line which is known to have short telomeres. Cawthon had measured TL for K562 which consists of 5290 base pairs in 2009 using Southern Blotting (Cawthon, 2009). K-562 acted as an internal control and calibrator sample in each run when samples are analysed with RT-qPCR. The average take-off value for K562 DNA for telomere run for the study was 10.38 ± 0.27 with a CV of 2.12%. The average take-off value for K562 DNA for single copy gene run for the study was 18.85 ± 0.25 with a CV of 1.33%. These values indicate consistency of the calibrator sample efficiency through out all the runs performed for the samples in this study.

Reproducibility of the TL assay was tested randomly by repeating any run on a separate occasion. The inter-assay coefficient variation (CV) was determined by using the formulae: (standard deviation/mean) x 100%. The inter- assay CV for TL assay in this study was 4.86%. Cawthon had observed an inter-assay CV of 5.8% (Cawthon, 2002). The CV result in our study indicates that the assay variation is low, in keeping with other published data (Refer to Section 1.2 and 1.3) and the result from the assay is reproducible.

A linear regression model between the data obtained from Tel run (see Figure 20) and SCG run (see Figure 21) measurements repeated on 2 separate occasions showed a strong correlation. The same data was used to calculate TL (T/S ratio) that showed significant correlation with R^2=0.89 and $p< 0.01$ (see figure 22).

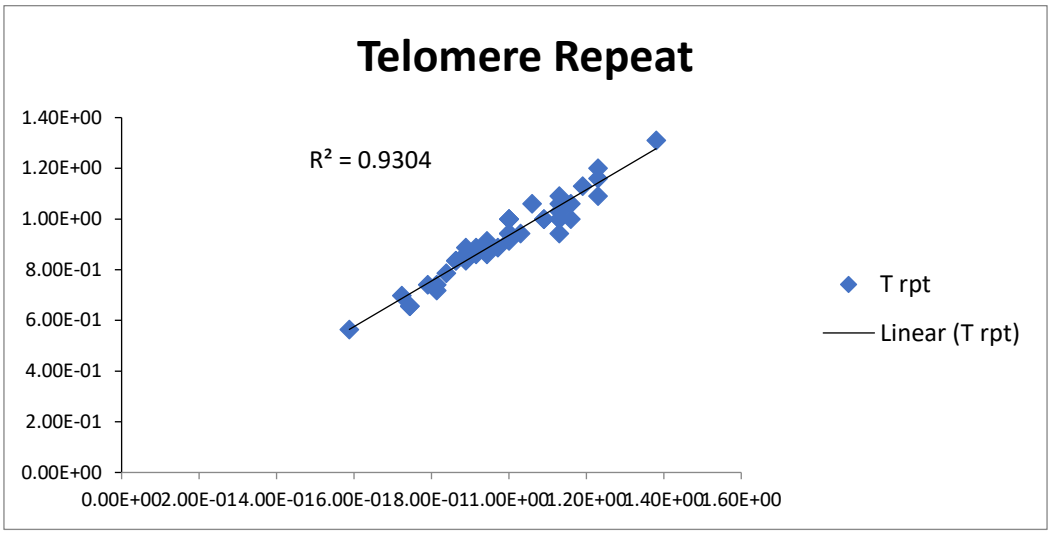

Figure 20: 48 samples from Tel run repeated on 2 separate occasions

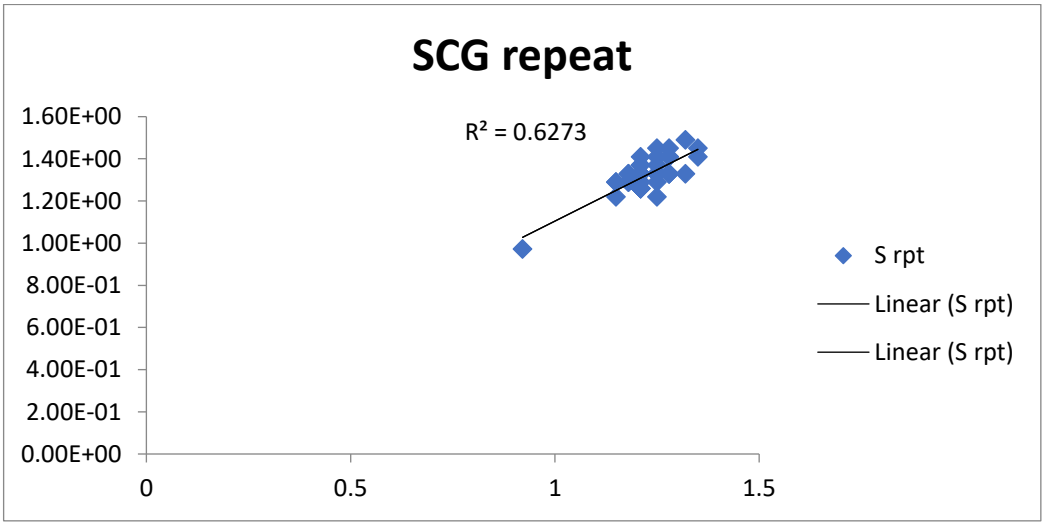

Figure 21: 48 samples from SCG run repeated on 2 separate occasions

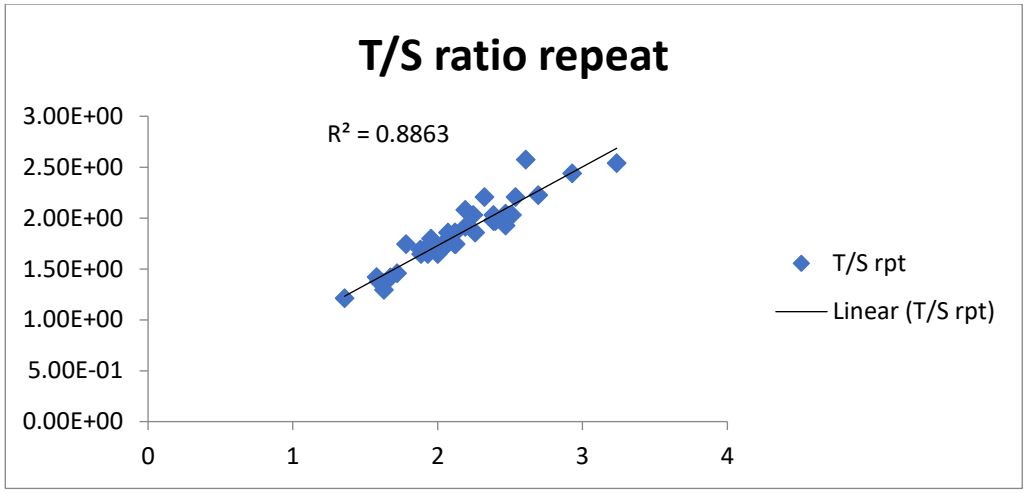

Figure 22: T/S ratio for 48 samples calculated based on repeat Tel and SCG paired runs from 2 separate occasion.

CHAPTER 5: LABORATORY METHODS ON DNA METHYLATION

5.1 DNA methylation methodology

There are 3 different recognised methods of measuring DNA methylation status. The choice of method selection is based on the aim of either the discovery of unknown epigenetic change or assessment of DNA methylation status within a particular region/gene of interest.

The methods described in measuring DNA methylation are:

A) Sodium bisulphite conversion and sequencing which is used in gene specific/GWAS studies. It is also the current gold standard in measuring DNAm status. Target BS sequencing is based on prior selection of predefined genomic regions of interest which have been identified by PCR amplification whereby the region of interest was identified from Whole Bisulphite Sequencing Data (See Figure 23).

B) Differential enzymatic cleavage of DNA which is used in locus specific analysis

C) Affinity capture of methylated DNA used in gene specific/GWAS studies.

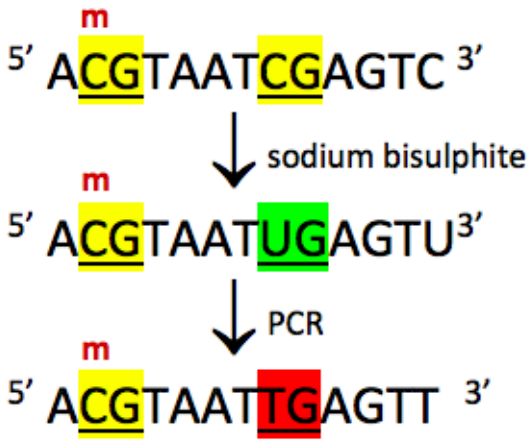

Figure 23: DNA sodium bisulphite conversion

Step 1: Denaturation of the template, Step 2: Sodium bisulfite deaminates cytosine residues, Step 3: Desulphonation removes the sulfite moiety to generate uracil, Step 4: Subsequent PCRs convert the Uracil to Thymines

The technique chosen for this PhD project was the sodium bisulphite conversion and sequencing as the candidate gene of interest were known, the number of samples to be analysed were large, speed, single nucleotide resolution, the amount of DNA/input material required is small, low cost of simultaneously assaying multiple CpG loci in multiple DNA samples with high reproducibility and the technique provided the highest level of coverage and resolution. Whole genome bisulphite sequencing is the gold standard method for mapping DNA methylation status.

5.2 DNA methylation methodology based on targeted bisulfite (BS) amplification sequencing using the Fluidigm Access Array (IFC)

5.2.1 Assay Design

The assay for measuring DNA methylation was performed with the assistance of Barts and The London Genome Centre; part of Queen Mary University of London. Methylation assays for this project was designed manually using the Pyromark assay design software or by running a script which uses 'R package', Bismark and Python Primer design software to output the primer sequences. A list of desired CpG sites for the study were sent to the Genome Centre for primer design. The gene names and chromosome position coordinates were identified from Hannum's database to design the assay. Unfortunately, only 44 out of 77 CpG sites had passed the initial design tests. The algorithm used in the primer design were unable to identify a pair of primers that would amplify the desired portion of the remaining 33 CpG sites of the genome within a pre-specified range of stringency. Therefore, we had to reduce the design stringency which subsequently enabled us to design primers for all the CpG sites but had to accept that in doing so, we will be increasing the likelihood that a proportion of the PCR reactions might fail. Primer pairs usually amplify portions of DNA containing multiple CpG sites. Therefore, by overlapping the PCR product of the primer pairs, we were able to deduce the methylation status at individual sites.

5.2.2 Sample quality control

All extracted DNA samples are assessed for DNA integrity using gel electrophoresis (Refer to Section 4.4; DNA integrity using Gel Electrophoresis). Then the DNA samples are quantified using the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, Ca, USA) with the dsDNA BR reagent kit. DNA is then diluted in 45uL of Tris-EDTA to give a final concentration of 500ng. Any samples below the minimum 500ng input was excluded.

5.2.3 DNA Bisulfite Conversion

Bisulphite conversion method involves treating DNA with bisulfite which converts unmethylated cytosine into uracil whilst methylated cytosine remains unchanged during the treatment (See Figure 23).

Bisulphite conversion of DNA was performed with the 96-well plate using the Zymo EZ DNA Methylation^{TM} Kit (Catolog Nos. D5001 & D5002, Cambridge Biosciences). The following protocol and kit converted non-methylated 'C's to 'T's through a 3-step process. An alternative incubation conditions recommended in the manufacturers guide was chosen for the bisulphite conversion for this project, which was 95^{o}C for 30 secs, 50^{o}C for 60 mins for 16 cycles with a 4^{o}C hold.

The bisulphite converted DNA was measured by the NanoDrop 8000 spectrophotometer V2.0 using the RNA-40 setting (ThermoScientific, USA).

Following the sodium bisulphite treatment of the DNA samples, the methylation profile is then determined by PCR amplification followed by DNA sequencing.

5.2.4 Library Generation Using Fluidigm Access Arrays and sequencing

Targeted methylation amplicon libraries for each sample were prepared using the Fluidigm 48: 48 Access Array integrated fluid circuit (IFC) (Fluidigm, Amsterdam, Netherlands). The Fluidigm platform enables up to 192 methylation targets (2-4 assay pools per well) to be amplified on a single access array across 48 DNA samples.

The 48:48 Access Array Integrated Fluidic Circuits (IFC) enables target enrichment of 48 samples at one time. 68 primer pairs targeting 68 CpG sites were generated with commons sequence tags (CS1- and CS2-). A 20x assay mix was prepared as per the manufacturers' protocol with assays at a final concentration of 1uM. Sample mix was prepared using 3uL of bisulphite converted DNA per sample. Bisulfite converted DNA is added to the Access array with the 48 primer assays and PCR mastermix. The primer inlets of the access array were loaded with pooled primer pairs (forward and reverse) diluted to a final concentration of 1uM per primer; on to the Pre-PCR IFC controller AX which loads the PCR components into the array chamber. PCR amplification is performed using the FC1 cycler with a custom programme as follows: 70^{o}C for 20min, 95^{o}C for 10min, [95^{o}C for 15secs, 60^{o}C for 30secs, 72^{o}C for 1 min] x 10 cycles, -1^{o}C touchdown, [95^{o}C for 15secs, 51^{o}C for 30secs, 72^{o}C for 1 min] x 29 cycles, 4^{o}C hold. This allows the fluidigm CS1 and CS2 custom sequences to be added

to the 5' end of each primer (CS1 is added to the forward and CS2 is added to the reverse), allowing for the later addition of unique barcodes to each sample allowing for PCRs from the same DNA sample to be pooled together. Each of the 48 sample inlets were loaded with 4uL of bisulphite converted DNA and mastermix solution. Once prepared the Access array was place in the primed pre-pcr IFC controlled AX for the samples and primers to be automatically combined over a 1.5-hour period. The array was then transferred to the Fluidigm FC1^{TM} Cycler for target amplification by PCR.

Following amplification, 1ul of the harvested enriched PCR product is run on an Agilent 2200 Tapestation and D1000 screentape (Agilant Technologies, Waldbronn, Germany) to assess the product size after which they are recovered and transferred to a 96-well PCR plate. This was only done for a random subset of samples as a quality control measure to determine the quantity and integrity of the PCR product. All the samples exhibited a product at the expected size.

All harvested samples/PCR products were than barcoded (with a unique Fluidigm barcode) using a second PCR amplification reaction and were taken forward for sample-specific barcoding using the Access Array Barcode Library for Illumina Sequencers (Fluidigm, Amsterdam, Netherlands). The function of this step was to label all amplicons form a given sample with a common sequence to allow subsequent identification once all of the PCR products had been pooled for sequencing. In addition, the barcode contains an Illumina sequencing adaptor (p5 and p7) that allows the final library of PCR products to bind to the oligo lawn of the Illumina Flow Cell as well as the read 1 and read 2 primer sequences (PE1 and PE2). The same samples

from harvest quality control were re-run on an Agilent Tapestation D1000 tape post barcoding to ensure a shift in size indicating successful ligation of index sequences and that the barcode has been incorporated. A successful reaction will generate a product ~60bp larger than the PCR product (See Figure 24).

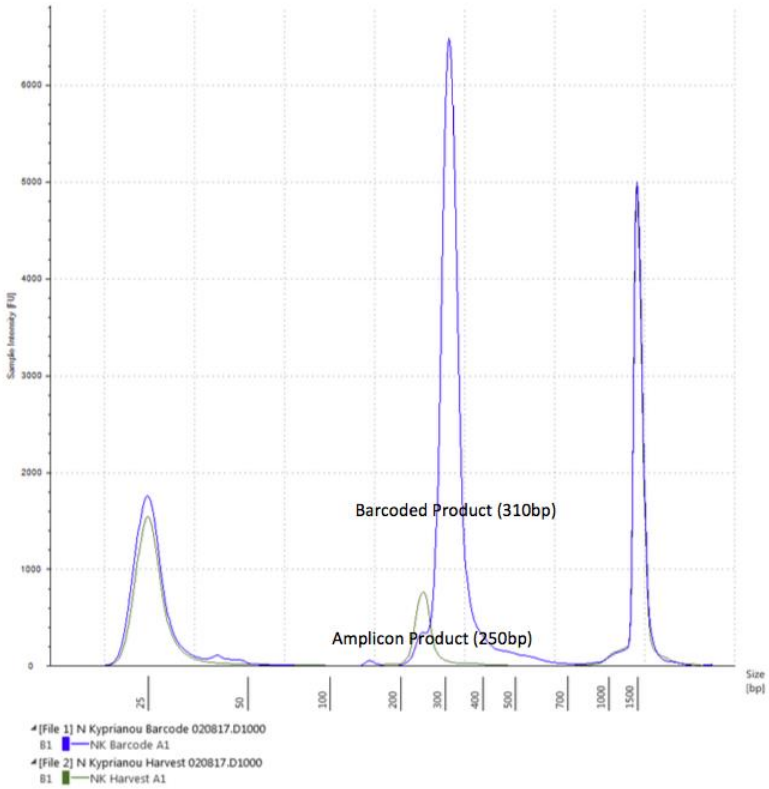

Figure 24: Library QC using the Agilent Tapestation D1000 tape - PCR product to Barcode product

An equal volume of each uniquely barcoded sample was combined to create one pooled library which was purified using Ampure XP beads at 1x ratio. The final cleaned library was quantified using the Qubit® 2.0 Fluorometer (Life technologies, Carlsbad, CA, USA) and the Agilent 2200 Tapestation with D1000 screentape (Agilent Technologies, Waldbronn, Germany). Once the PCR products had been successfully barcoded, they were pooled for sequencing using the Illumina MiSeq® v2 300 cycle kit to generate 150bp paired end reads.

The sequencing process generates millions of reads in total representing all of the DNA fragments. Sequences within the pooled DNA library are separated into individual samples based on the unique sample specific barcodes attached to each fragment during preparation of the library. Within fragments from the sample, reads with similar base sequences are clustered and grouped by forward and reverse reads. Any discrepancies are resolved by comparison within the grouped reads. Finally, the percentage of methylated cytosine residues is calculated for each CpG site for all fragments generated from a single sample.

5.3 DNA methylation analysis

The proportion of methylation at a particular CpG site is referred to as a beta value which can change over a course of one's lifespan. To understand the nature of DNA methylation based (DNAm) age estimators, it is necessary to appreciate what is being measured. Each CpG clock takes a value between 0 and 1, whereby 1 indicates methylated and 0 indicates unmethylated. Importantly, the values of the clock CpGs (and the vast majority of all CpGs) are almost never 0 or 1 but a value in between. For example, a value of 0.66 indicates that 66% of the thousands of copies of that particular CpG, which were derived from thousands of cells, are methylated.

To define an ageing methylation signature, a similar approach to that of both Hannum et al. (Hannum *et al.*, 2013) and Horvath (Horvath, 2013) was used. All the analysis was restricted to those CpGs which were covered by > 100 reads to reduce the effect of highly noisy methylation values, leaving a total of 316 CpGs. A model was initially built from the data provided by using 27 "healthy" control samples with known ages. Three samples were removed due to low amount of sequencing leaving 24 samples to build a model on. Ages in years were transformed using the following function as suggested by Horvath (Horvath, 2013).

$$f(A) = \begin{cases} \log(A+1) - \log(A+1), & A \leq 20 \\ \dfrac{A-20}{A+1}, & A > 20 \end{cases}$$

Where *A* is the age of a sample in years. The rationale for this transformation is that it is

a) Continuous

b) Logarithmic up till 20 years old and then linear from that point onwards

c) Is defined for prenatal samples e.g. A can be -1.

Following this conversion, an elastic net regression model was used to regress the 316 CpGs and then a prediction based on the methylation levels of each of these 316 CpGs can be made. As part of the elastic net regression a single hyper-parameter is fitted using a 5-fold cross validation. To test the model, we used a leave one out cross validation. Specifically, we removed a single sample and trained the model as above and then predicted the age of the missing sample. We did this for each sample and calculated the RMSE of the prediction resulting in a RMSE of 11.59 years.

To see if we could improve this prediction we used existing Illumina 450K data provided by Hannum et al. (n=656)(Hannum *et al.*, 2013) . First, we overlapped the 316 CpGs with probes contained on the array leaving a total of 49 CpGs in common between the two experiments. The CpG ID of these is provided in the file probes_used.csv (Refer to Appendix H: CpG ID's of Probes Used in Measuring DNA Methylation Status). Following the same approach as above an elastic net regression model was used to regress the 49 CpGs. The hyper-parameter was fitted using a 10-fold cross validation (due to larger number of samples available) and the model was trained on the Hannum et al data tested on the 24 "healthy" control samples. This resulted in a reduced RMSE of 6.60 years and a spearman correlation of 0.76 (See Figure 25).

The model was than run on the remaining samples to predict the ages of each sample. A 95% prediction interval based on the sample distribution of the 24 samples was calculated.

A measure of disproportionate ageing; in the form of age acceleration and apparent methylomic ageing rate are calculated for each study participant and subsequently used as variables for data analysis. DNAm delta age is the difference between DNA methylation age (derived from the model) and chronological age (derived from patients' DOB at the time blood samples were taken). Whilst apparent methylomic ageing rate (AMAR) is calculated by dividing DNA methylation age by chronological age. AMAR greater than 1 is interpreted as fast ageing whereas AMAR less than 1 represents slow ageing (Hannum *et al.*, 2013).

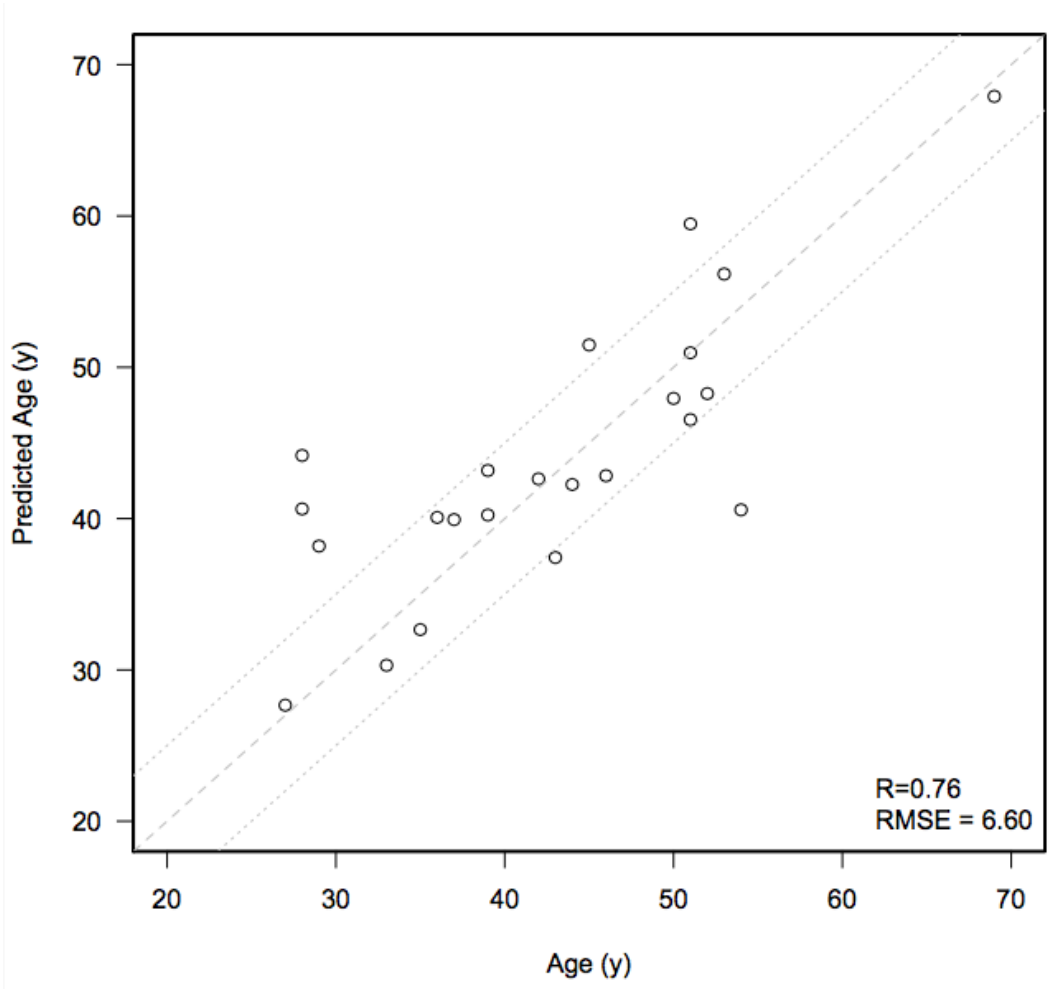

Figure 25: Predicted age in years against actual age in years for the 24 healthy controls. Grey dashed line is y=x, or a perfect prediction. Double dashed lines are +/- 5 years.

It is not surprising that the DNAm age is an excellent correlation of chronological age as the model is built based on healthy donors chronological age.

CHAPTER 6: RESULTS ON TELOMERE LENGTH, DNA METHYLATION AND FRAILTY IN STUDY COHORT

6.1 Summary of background demographics of study recruits at baseline

A total of 339 patients were recruited for the study during the period of December 2015 to July 2018 from Barts Health NHS Trust dialysis units and outpatient clinics. Table 5 outlines the details of the three different cohorts of patients recruited for the study and distribution of their various characteristics (where data was available). Patients on dialysis were grouped into haemodialysis (HD) and peritoneal dialysis (PD) according to their respective modality at the time of recruitment. Potential live donors were considered as control.

Characteristics	**Haemodialysis (n= 125)**	**Peritoneal Dialysis (n= 114)**	**Control (n= 100)**	**P-values**
Age (mean ± SD)	53.2 ± 13.6	55.7 ± 14.2	44.5 ± 13	**<0.001**
Female (n, %)	44 (35.2)	43 (37.7)	56 (56)	**0.004**
Ethnicity (n, %)				
White/Caucasian	64 (51.2)	34 (29.8)	58 (58)	**<0.001**
Asian	29 (23.2)	52 (45.6)	27 (27)	
Black/Afro-Caribbean	30 (24)	27 (23.7)	12 (12)	
Mixed/Other	2 (1.6)	1 (0.9)	3 (3)	
Cause of ESKD (n, %)			NA	**0.006**
Diabetes	24 (19.2)	49 (43)		
Hypertension/Renovascular	21 (16.8)	11 (9.7)		
Glomerulonephritis	36 (28.8)	26 (22.8)		
Cystic/hereditary kidney	13 (10.4)	7 (6.1)		
Pyelonephritis/interstitial nephritis	10 (8)	7 (6.1)		
Others including unknown	21 (16.8)	14 (12.3)		
Pre-existing cardiovascular disease (n, %)	31(24.8)	31(27.2)	0 (0)	0.784
Diabetes (n, %)	35 (28)	55 (48.2)	0 (0)	**0.002**
Previous Cerebrovascular even (n, %)	11 (8.8)	8 (7)	0 (0)	0.788
Charlson's Comorbidity Index (n, %)				
Score ≤ 2	64 (51.2)	50 (43.9)	100 (100)	0.462
Score 3	21 (16.8)	15 (13.2)		
Score 4	21 (16.8)	25 (21.9)		
Score 5	13 (10.4)	14 (12.3)		
Score >5	6 (4.8)	10 (8.8)		

BMI (mean ± SD)	26.9 ± 5.4	27.6 ± 5.1	26.6 ± 4	0.274
Smoking (n, %) (yes/no/former smoker/unknown)	18/64/28/15 (14.4, 51.2, 22.4, 12)	7/57/39/11 (6.1, 50, 34.2, 9.7)	20/62/14/4 (20, 62, 14, 4)	**0.047**
Dialysis access (n, %)			NA	NA
Arteriovenous fistula	73 (58.4)	NA		
Catheter	47 (37.6)	108 (94.7)		
Unknown/missing data	5 (4)	6 (5.3)		
Dialysis vintage in weeks (median, IQR)	162.07 (75.1, 296.9)	79.78 (30.8,137.8)	NA	**<0.001**
Haemoglobin (mean ± SD)	11.17 ± 1.30	10.79 ± 1.72	NA	0.056
White cell count (mean ± SD)	6.99 ±2	7.77 ± 2.26	NA	**0.005**
Albumin (median, IQR)	42 (40,45)	39 (37,42)	NA	**<0.001**
C-reactive protein (n, %)			NA	0.869
<5	70 (56)	66 (57.9)		
≥5	55 (44)	48 (42.1)		

Table 5: Basic demographics of all patients recruited for the study.

Data presented in the form of n=number, %= percentage, mean ± standard deviation (SD) for normally distributed data and median with interquartile range (IQR) for skewed data. All significant results in bold. Significance testing was not conducted in the group with unknown smoking.

Table 5 summarises the basic demographic data and variables of interest from the study recruits. The score derived from the Charlson's comorbidity index (CCI) was grouped into 5 categories based on their scores as depicted in the table above. C-reactive protein (CRP) was treated as a categorical variable based on their values. All

categorical variables which include; gender, ethnicity, cause of ESKD, pre-existing cardiovascular disease, diabetes, pre-existing cerebrovascular disease, Charlson's comorbidity index, smoking and CRP were tested for significance between groups by using the Chi Square test. Age and BMI where analysed with ANOVA whilst haemoglobin (Hb) and white cell count (WCC) were analysed using 2 sample T-test. Both dialysis vintage and albumin results were skewed and were analysed with Mann Whitney U-test. All significant difference in variables between groups are in bold.

6.1.1 Age distribution among study cohorts

There was a total of 239 patients on renal replacement therapy (RRT) with an age range of 23 to 83 years of age. There were 100 patients consisting of healthy potential live donors in the control group with an age range between 20 to 75 years of age. The mean age and standard deviation (SD) of the case cohort was 54.4 ± 13.93. There was no difference in age between the haemodialysis and peritoneal dialysis groups, ($p=0.18$) using a 2 sample T-test. There was a total of 125 patients on chronic haemodialysis with an age range of 23 to 80 years among the study recruits. There was a total of 114 patients on peritoneal dialysis recruited for the study with an age range of 23 to 83 years. There was significant difference in ages between the two-dialysis groups and healthy control, $p<0.01$. This is due to the fact that the healthy control group which consists of potential donors are more likely to be younger with a mean age of 44.5 ± 13 in comparison to the case group which had a mean age of 54.4 ± 13.9 years.

6.1.2 Gender distribution among study cohort

Table 5 shows that the gender distribution among the three groups of patients in this study were significant, $p<0.01$ but there was no significant difference between gender in the case group consisting of HD and PD patient, $p=0.787$.

6.1.3 Dialysis vintage distribution in case groups

Dialysis vintage was defined as the duration of time spent on dialysis treatment in weeks. Data on dialysis vintage distribution was only available in 226 patients with a range of 2.1 to 1367.3 weeks. The dialysis vintage in the HD group ranged from 2.1 to 1367.3 weeks with a median of 162 weeks (Interquartile range: 221.8). The PD group had a dialysis vintage range of 7 to 546.7 weeks with a median of 79.8 weeks (Interquartile range: 107). Patients on HD had a significantly longer dialysis vintage than patients on PD, $p<0.01$. This difference is likely due to the nature of the two modalities whereby prolonged exposure to PD is associated with failure of this treatment in the long run. In fact, most patients on PD are electively switched to HD after five years due to risk of encapsulating peritoneal sclerosis in the current recruitment renal unit, if deemed appropriate. Younger patients also usually opt for PD due to lifestyle factors and often transition into the mode of transplantation.

6.2 Frailty at baseline and one year follow up

Completed frailty assessments at baseline were available for 299 patients in total; 213 (71.2%) dialysis patients and 86 (28.8%) healthy controls. Frailty scores were calculated as outlined in 'Chapter 3: Clinical Methods' for each domain and a final score out of five determines ones frailty status. Those with a total score of ≥3 were considered frail and scores <3 were not frail. Dialysis patients were followed up for 12 months and had repeat frailty assessments done.

6.2.1 Frailty in healthy control and dialysis

There were no frail patients in the healthy control group which was expected as these patients consisted of potential live donors. Table 6 outlines the distribution of frailty between control and dialysis cohort.

Frailty level	**Control (n=86)**	**Dialysis (n=213)**	**P value**
Not frail (n, %)	86, 100	143, 67.1	**< 0.001**
Frail (n, %)	0, 0	70, 32.9	

Table 6: Difference in Frailty status in control and dialysis group

Therefore, the subsequent sections will only be focusing on frailty status in the dialysis cohort.

6.2.2 Frailty and gender

Table 7 shows the gender distribution of frailty status among the dialysis cohort. A total of 78 females completed the frailty assessment at baseline and 21 females (26.9%) were considered frail. The relative risk of frailty was 1.078 which shows that females were 7.8% more likely to be frail than men whose odds of frailty was increased by 6.3%.

	Frail (score ≥3)	Non-frail (score <3)	**Total**
Male (n, %)	49 (36.3)	86 (63.7)	**135**
Female (n, %)	21 (26.9)	57 (73)	**78**
Total (n, %)	**70 (32.7)**	**143 (67.3)**	**213**

Table 7: Frailty distribution and gender

A Pearson's Chi-square test was used to compare the frequency of frailty in men and women. There was no significant interaction found for gender and frailty using a Chi square test with a p=0.860.

6.2.3 Frailty and age

Frailty data were available from patients aged 23 to 83 years. The image below shows the age distribution in each category of frailty based on their total scores from five different domains.

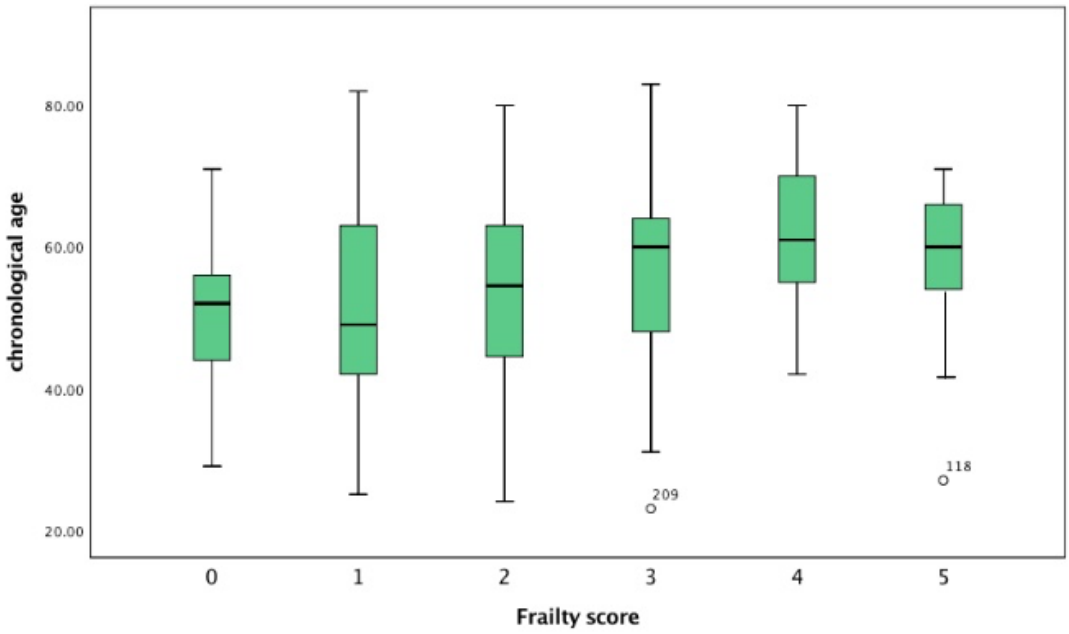

Figure 26: Age distribution among frailty score of 0 to 5 in the dialysis cohort

Frailty and age were strongly correlated in this study, this association was significant $R=0.249$, $p<0.01$.

6.2.4 Frailty among haemodialysis and peritoneal dialysis

Table 8 outlines frailty scores between the two dialysis cohorts. The relative risk of being frail was 1.69 times higher in the haemodialysis cohort in comparison to peritoneal dialysis, p=0.012**.** The differences in frailty status between the two groups was significant, p=0.015. There may be an effect of longer dialysis vintage in the HD group contributing to this significance.

Modality	**Frail (score ≥3)**	**Non-frail (score <3)**	**Total**
Haemodialysis (n, %)	45 (40.9)	65 (59.1)	**110**
Peritoneal dialysis (n, %)	25 (24.3)	78 (75.7)	**103**
Total	**70**	**143**	**213**

Table 8: Frailty score in 2 different dialysis modalities

6.2.5 Frailty component data

Figure 27 shows the distribution of frailty components among the 110 HD patients and 103 PD patients who completed their frailty assessments.

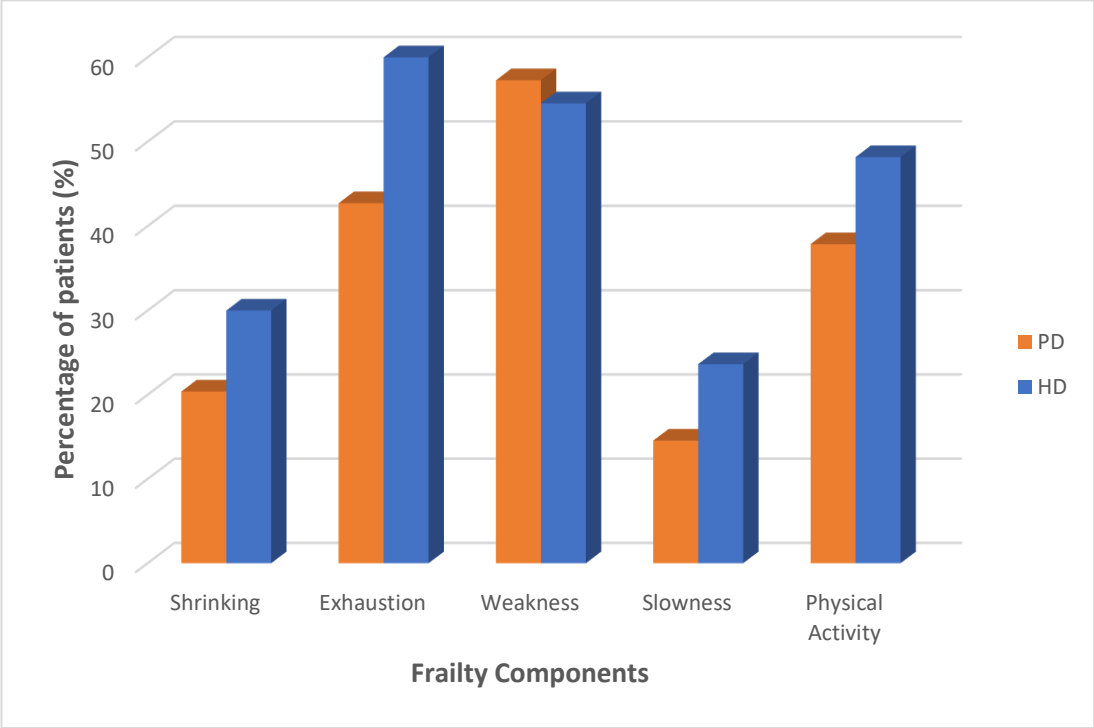

Figure 27: Frailty components in dialysis study cohorts at baseline

A univariate logistics regression was conducted to assess each of the frailty components and modality with HD as a reference (See Table 9). Exhaustion was less in the PD cohort in comparison to the HD with an OR of 0.497, p=0.012. This may be related to the fact many HD patients reported exhaustion associated with dialysis treatment in hospital on average thrice weekly leading to fatigue which affected the way they felt or behaved.

Frailty component	Odds ratio	Standard Error	95% confidence interval		P values
			Lower bound	Upper Bound	
Shrinking	0.598	0.321	0.318	1.121	0.109
Exhaustion	0.497	0.278	0.288	0.858	**0.012**
Weakness	1.117	0.276	0.650	1.920	0.688
Slowness	0.551	0.358	0.273	1.112	0.096
Physical activity	0.655	0.279	0.38	1.132	0.129

Table 9: Univariate analysis between frailty components and dialysis modality.

6.2.6 Difference in frailty scores at baseline and at 1 year follow up

Dialysis patients were followed up at 12 months and had repeat frailty assessments. Only 155 patients (58.7% from HD group) had repeat frailty assessments completed. Out of the 155, 86 patients (53.5% from HD group) remained on dialysis as modality for renal replacement therapy and 69 patients had a kidney transplant during the first year of follow up which will be discussed separately in Section 6.2.7.

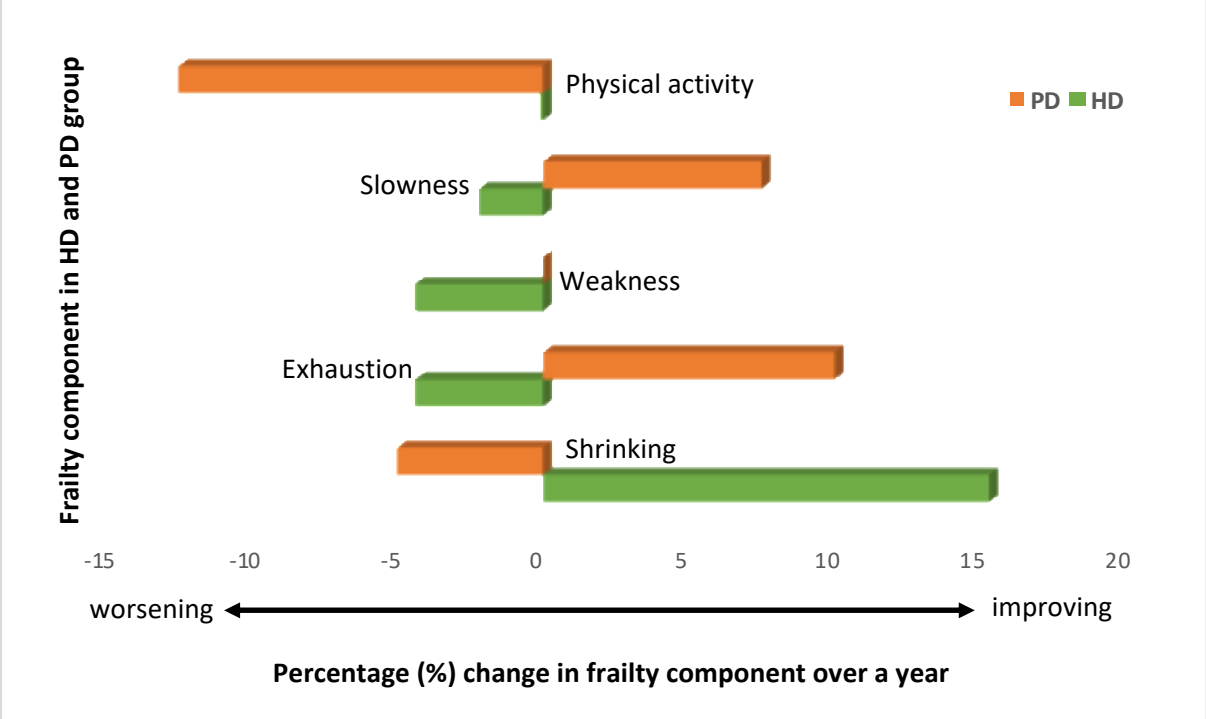

Figure 28: Changes in frailty component from baseline to year 1 in patients remaining on dialysis according to modality

Changes in frailty components were calculated as percentage change from baseline therefore, percentage of a frailty component at baseline minus percentage of frailty component at 1 year follow up. A positive result indicates improvement in the frailty component and vice-versa.

Figure 28 shows that slowness and exhaustion improved in the PD group but physical activity and shrinking worsened at the 1 year follow up. There was no change observed in weakness. For the HD group, only shrinking had improved but weakness, slowness and exhaustion had worsened. No change was seen in physical activity at the 1 year follow up in the HD group. Shrinking as reflected by weight may have improved due to improvement in appetite or better diet. However, there is always a difficulty in establishing shrinking over a year as assessment of weight in dialysis patients are affected by fluid weight and changes in fat to muscle composition. There is a drive to using alternate measures of weight in dialysis patients e.g. body composition monitor. There are also increasing attempts and efforts to develop and validate other frailty tools such as comprehensive geriatric assessments, Clinical frailty scale, FRAIL and frailty assessment for care planning tool (Clark *et al.*, 2017) (Nitta, Hanafusa and Tsuchiya, 2017). (Refer to Section 8.1: Challenges and limitations with laboratory and clinical methodology).

Physical activity in the PD patients had deteriorated and the reason for this may be due to changes in the dialysis regime limiting one's activity level. The study did not take into account episodes of PD peritonitis which may have contributed to change in dialysis regime and dialysis adequacy which could affect physical activity directly and indirectly (See Figure 7 on cycle of frailty).

There was no significant difference in change over a year between the five frailty components. Grip strength at baseline and at 1 year was also not different, p=0.612. There was no significant change in overall frailty scores at baseline and at 1 year follow up in either of the HD or PD group, p=0.357.

6.2.7 Difference in frailty score baseline and 1 year post renal transplant

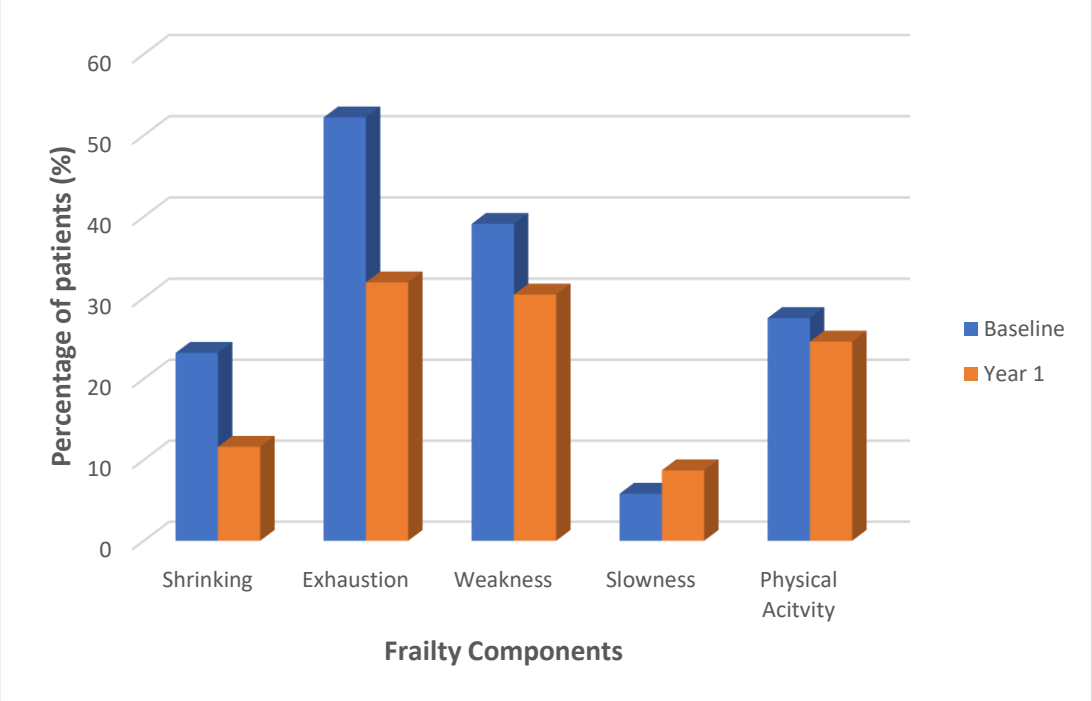

Figure 29: Distribution of frailty components in patients who had renal transplant during study follow up. Baseline data from dialysis patients recruited for study and year 1 follow up post renal transplant.

Frailty was measured at baseline and 1 year in 69 dialysis (45 HD, 24 PD) patients who subsequently had a renal transplant during the study period. The study was unable to account for the exact time of the renal transplant but all patients with 1-year frailty assessments had their renal transplant for at least three months minimum. The frailty components that have improved in this cohort are shrinking, exhaustion and weakness (See Figure 29). Physical activity level remained fairly similar at both time points (See Figure 29). However, exhaustion was the only component that was significantly different from baseline to 1 year, p=0.007 which may represent a better quality of life and energy levels with renal transplantation.

6.3 Baseline Telomere length analysis in the study cohort

TL was measured in the form of T/S ratio. Therefore, results on TL are presented in this form i.e. T/S ratio in this section. T/S ratio was measured in 239 dialysis patients and 100 healthy controls recruited for the study. However, three samples were excluded from the dialysis group and one from the control group for further analysis, as the take-off value difference between the duplicate repeats in the run was >0.2 or two cycle difference. Therefore, T/S ratio results were available for 335 patients (124 HD, 112 PD, 99 control) for further data analysis.

6.3.1 Telomere length and age

It is widely known that TL declines with age. This association was observed in the current study cohort of 339 patients demonstrating a negative correlation between T/S ratio and age, r= -0.416, p<0.001 (See Figure 30). The correlation between T/S ratio and age in the dialysis population only (See Figure 31) remained significant, r= -0.403, p<0.001. (See Figure 31) Both these results remained significant at p<0.001 even with gender as a co-variate in a multi variate regression analysis.

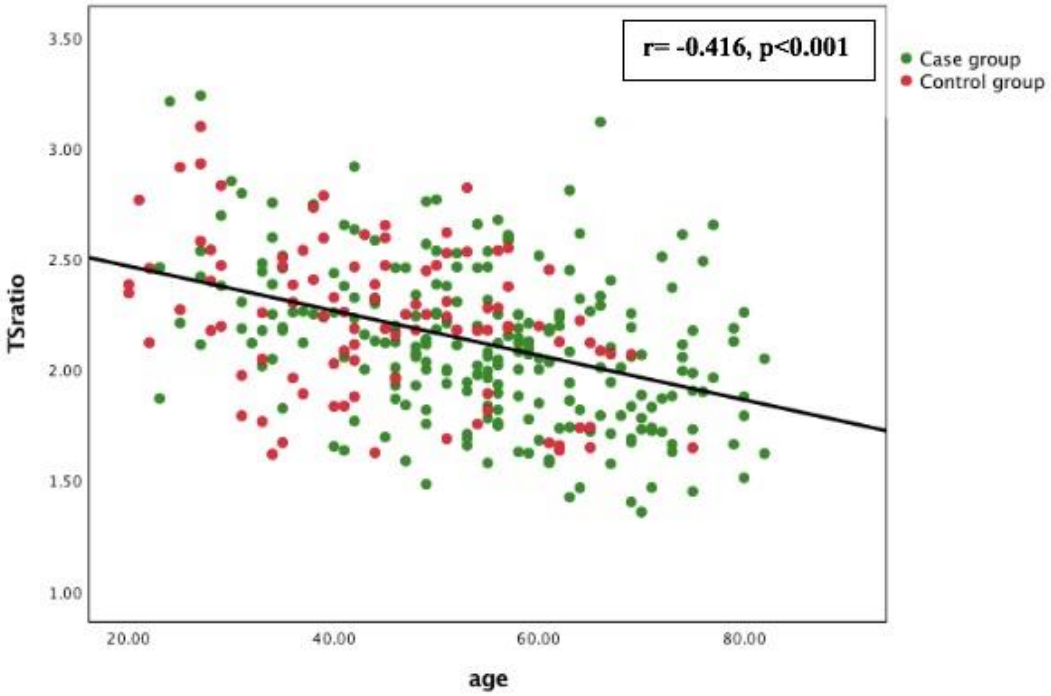

Figure 30: Correlation between T/S ratio and age in the study cohort (includes both cases and control).

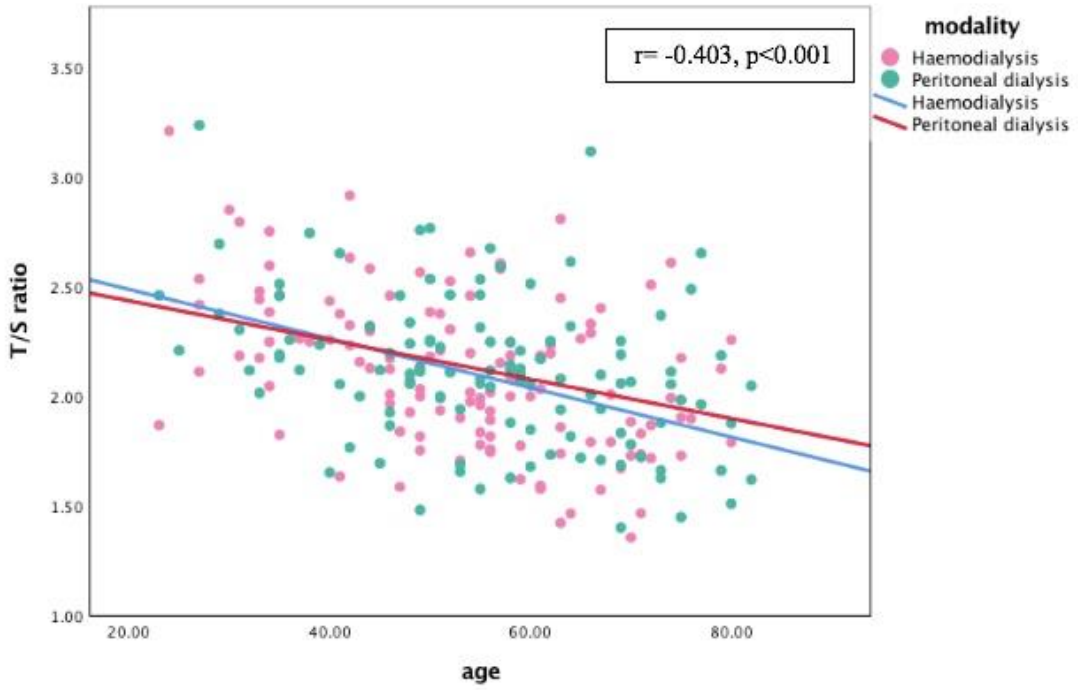

Figure 31: Correlation between T/S ratio and age between HD and PD group

6.3.2 Telomere length and gender among study cohort

The mean T/S ratio among male and females in the control group was 2.24 ± 0.39 and 2.22 ± 0.28 respectively (See Figure 32). There was no significant difference in mean T/S ratio between gender in the healthy cohort in our study p=0.778. However, mean T/S ratio was higher in females than in males among dialysis patients (See Figure 33). Females had a mean TL of 2.21 ± 0.38 in comparison to males who had a mean TL of 2.07 ± 0.31 from the dialysis cohort (See Figure 33). These differences were found to be significant, p=0.007. This significance diminished following a multivariate analysis of the data adjusting for age, p=0.077 (See Table 11).

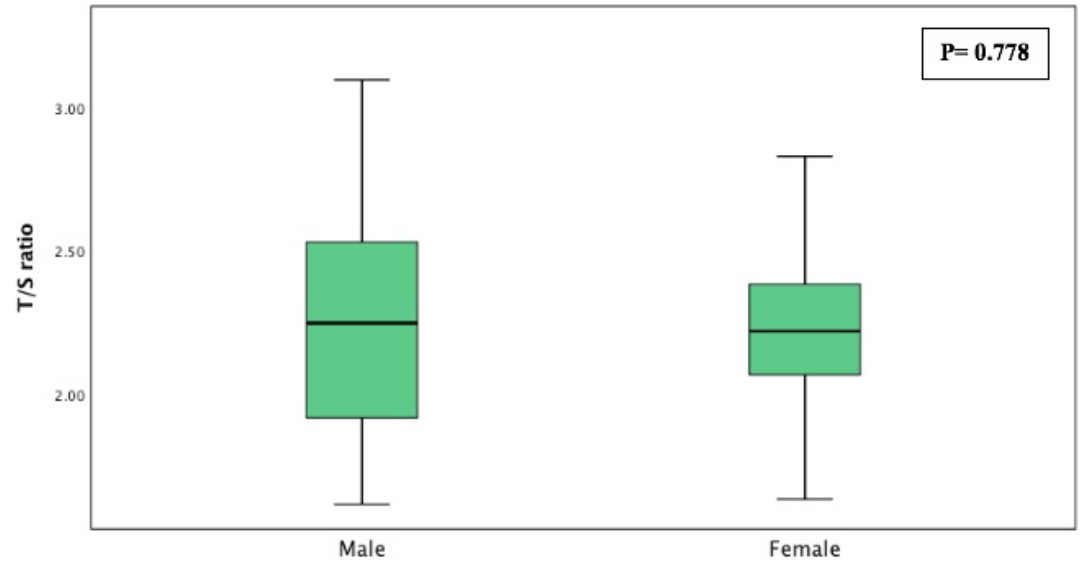

Figure 32: Whisker box plot showing T/S ratio (y-axis) distribution in male and female in the healthy control group. Horizontal line represents median value, box represents interquartile range and vertical line indicates range.

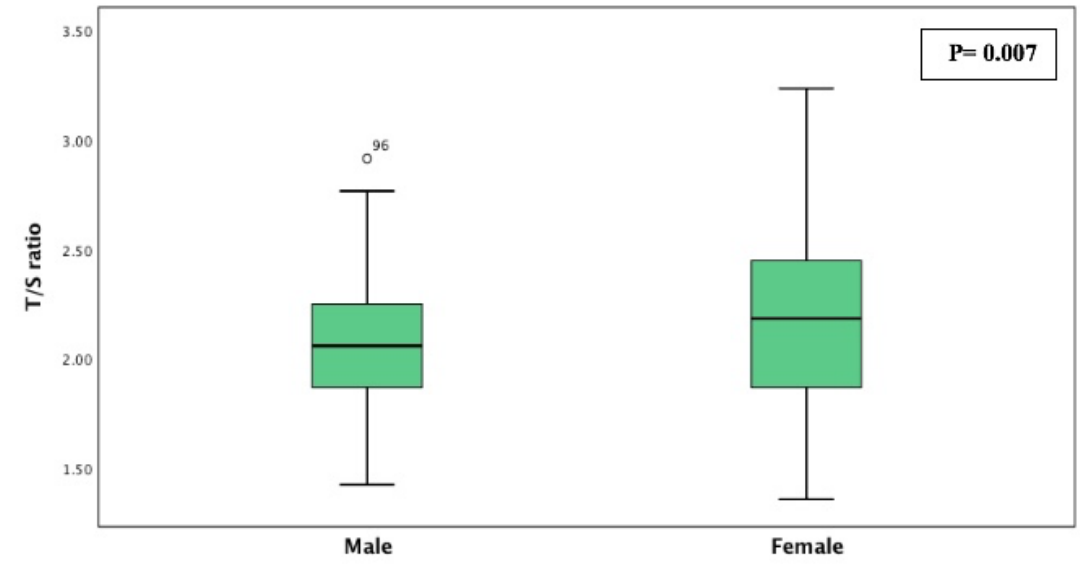

Figure 33: Whisker box plot showing T/S ratio (y-axis) between gender in the dialysis cohort. P=0.007 (unadjusted significance)

6.3.3 Difference in telomere length between dialysis (case group) and control group

Mean T/S ratio in the healthy control group was 2.23 ± 0.33 in comparison to mean T/S ratio in the dialysis group which was 2.11 ± 0.34 (See Figure 34). This difference was significant when analysed with a univariate linear regression model, p= 0.006. A multivariate linear regression model adjusting for age and sex revealed no further significance between the case group and control group, p=0.886.

Subsequent analysis was done to investigate if there was a difference between T/S ratio in the three different study groups. The mean T/S ratio in the control, HD and PD group were 2.23 ± 0.33, 2.11 ± 0.34 and 2.12 ± 0.34 respectively. A univariate linear regression model showed that T/S ratio between the three groups were significant, p=0.022 (See Figure 35). Table 10 shows that HD patients had a mean T/S ratio of less than 0.114 and PD patients had a mean T/S ratio of less than 0.113 in comparison to the control group.

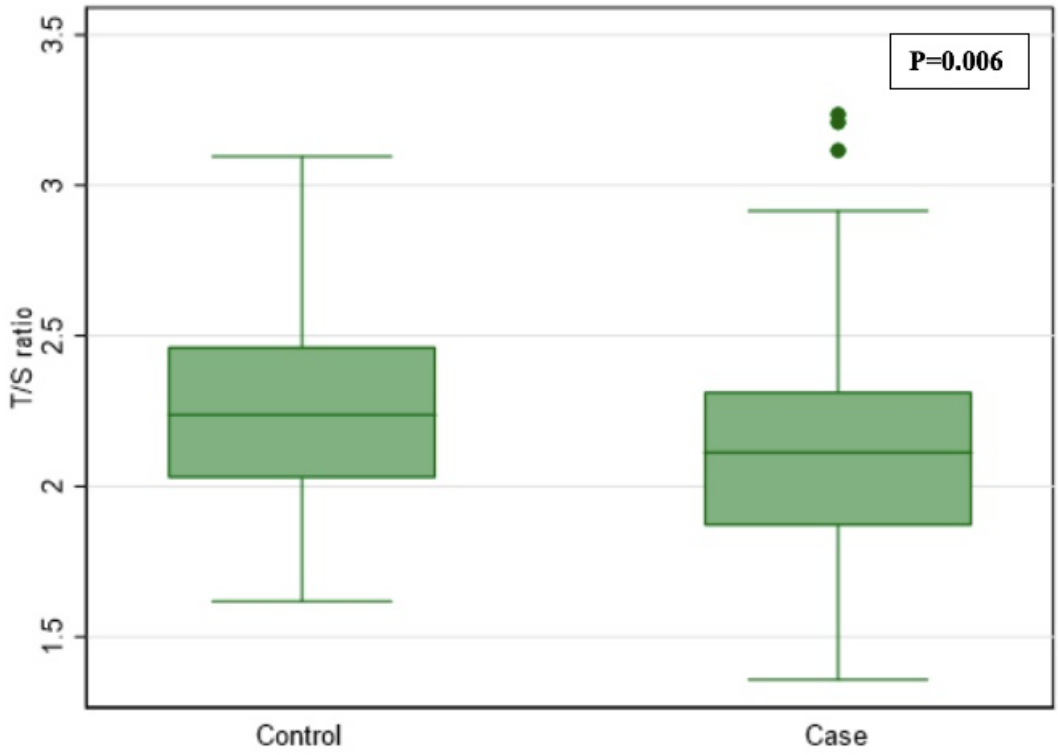

Figure 34: Whisker Box plots for T/S ratio in control and Dialysis groups.

T/S ratio	Estimate	Standard error	95% confidence interval		p-value
			Lower Bound	Upper bound	
HD	-0.0114	0.046	-0.204	-0.024	**0.014**
PD	-0.0113	0.047	-0.205	-0.021	**0.017**

Table 10: Univariate linear regression model to evaluate the relationship between T/S ratio and the 3 different groups with control group being the baseline.

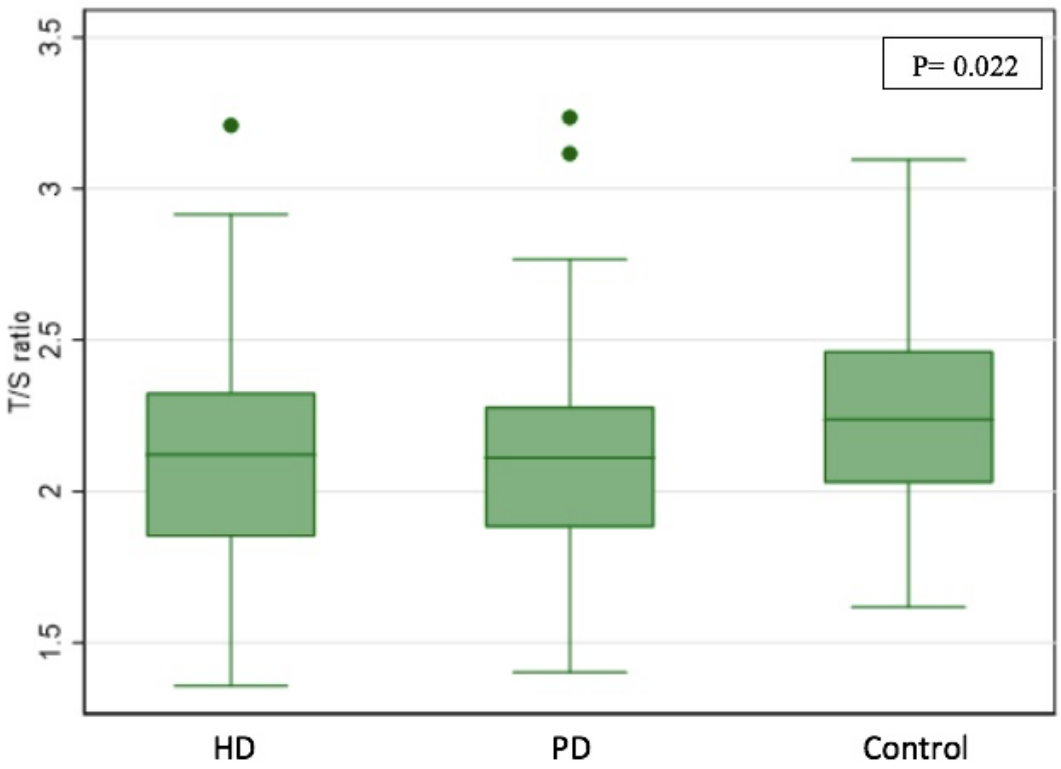

Figure 35: Whisker Box plots for T/S ratio in HD, PD and control group, P=0.022 (unadjusted by group)

However, this significance was lost following adjustment for age and gender in a multivariate linear regression model between the three groups (See Table 11). Table 11 shows that being on dialysis is not associated with T/S ratio after the data was adjusted for age and sex, p= 0.876 in PD and p= 0.708 in HD. The change in T/S ratio is reflected by the estimate, this was due to the dialysis group being older than the controls.

T/S ratio	Estimate	Standard Error	95% Confidence Interval		P-value
			Lower Bound	Upper Bound	
Age	-0.010	0.001	-0.012	-0.007	**0.000**
Female	0.062	0.035	-0.007	0.132	0.077
HD	-0.017	0.044	-0.103	0.070	0.708
PD	0.007	0.046	-0.082	0.097	0.876

Table 11: Results from multivariate linear regression model comparing dialysis and control groups adjusting for age and sex. No difference between TS ratio observed in gender and groups.

A multivariate linear regression analysis was then conducted to investigate any difference between the two dialysis groups. The analysis showed that T/S ratio in PD was **not** significantly different to HD, although the T/S ratios were on average 0.0225 longer in PD patients in comparison to HD patients following adjustment for age and sex (See Table 12).

T/S ratio	Estimate	Standard error	95% confidence interval		P Values
			Lower Bound	Upper Bound	
Female	.0890	.04273	.0048	.1731	**0.038**
Age	-.0096	.00149	-.0125	-.0067	**0.000**
PD	.0225	.04104	-.0584	.1034	0.584

Table 12: Multivariate Linear regression analysis between the 2 dialysis groups adjusted for age and gender

6.3.4 Telomere length and mortality in dialysis group

There was a total of 10 deaths during the follow up period (365 days). There was no loss of data in the follow-up dialysis groups. The T/S ratio were standardised and converted to Z scores to evaluate the probability of death for analysis. There was no significant association with ZT/S (standardised T/S ratio) and mortality observed following adjustment for age and sex (See Table 13).

Death	Odds ratio	Standard error	Z	P>[z]	95% confidence interval	
Age	1.0764	.0343	2.31	**0.021**	1.0112	1.1458
Female	.9090	.6594	-0.13	0.895	.2193	3.767
ZTS	.6899	.2680	-0.96	0.339	.3222	1.477

Table 13: Logistic regression model using Standardised T/S ratio (ZT/S) as a predictor for death.

However, the risk of death was 31% lower (1-0.689) per SD increase in TL. Therefore, the direction of effect is consistent with expectations, i.e. longer = less risk of death but the small number of events meant that this test was underpowered to detect a difference. A longer duration of follow up or a larger number of patients is required to evaluate the effect of T/S ratio and mortality outcome which may provide a higher number of events.

6.3.5 Telomere attrition over a 12-month period among dialysis patients

A total of 228 patients had repeat blood samples taken at 12 months but only 142 sample pairs were selected to investigate telomere attrition. There were 86 samples excluded for further analysis due to reasons as listed below:

1) There were no 12 months follow up frailty scores available
2) Patients who transitioned to a different dialysis modality
3) Any blood samples that were taken during period of illness requiring hospitalisations
4) Any repeat sample with evidence of compromised DNA quantity and quality assessed by spectrophotometry

These blood samples from baseline and year-1 (12 months from initial blood sampling) were analysed simultaneously i.e. the original samples were re-run side by side along with the year-1 sample to minimise the effect of potential technical variation associated with the assay for analysis. This would mean that the DNA of the baseline sample would have been stored for at least a year longer than the samples from year 1.

Out of 142 paired samples, only 136 paired samples were successfully measured. Failure of either sample from a pair meant that the sample had to be excluded from further analysis. Therefore, data on telomere attrition changes (ΔT/S) was reported for 136 patients with results in paired samples (79 HD, 57 PD). ΔT/S was calculated by deducting year 1 T/S ratio from baseline T/S ratio. There were 66 patients with a positive ΔT/S, 63 patients with a negative ΔT/S and 7 patients had no change in T/S ratio at baseline and year 1. Positive ΔT/S represented telomere shortening and negative ΔT/S would indicate telomere elongation. Mean ΔT/S was 0.0002 ± 0.23. A univariate analysis was conducted to investigate if ΔT/S differed in either of the dialysis modalities and no significance was found, p=0.129 (See Figure 36). A multivariate analysis adjusting for age and gender revealed a borderline significant, p=0.05 between modalities with PD group having mean difference in ΔT/S of -0.075 (See Figure 37 and Table 14). This would imply that the PD group have a slower rate of telomere attrition or underwent telomere elongation in comparison to the HD group.

The change in T/S ratio at baseline and year 1 was estimated with a mixed model approach (See Table 15) using patient ID as a random effect to estimate the change in standardised T/S ratio over time (not the ΔT/S) which showed no significant observed changes in standardised T/S ratio within 12 months, p= 0.992, age and gender remained significant in this model (See Table 15).

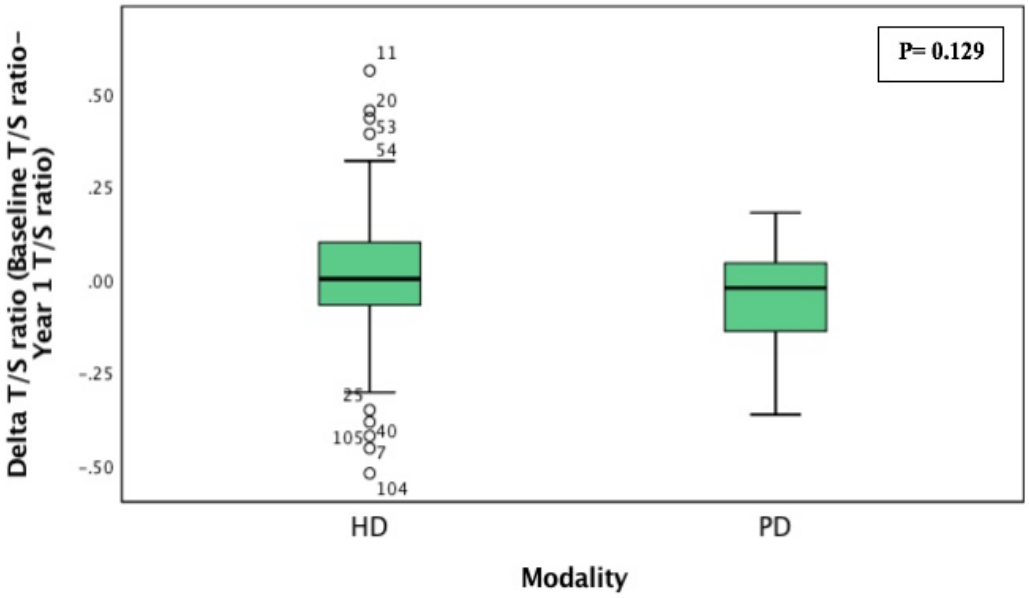

Figure 36: Delta T/S ratio between HD and PD group (all case group included).

Delta T/S ratio was calculated as baseline T/S ratio- year 1 T/S ratio.

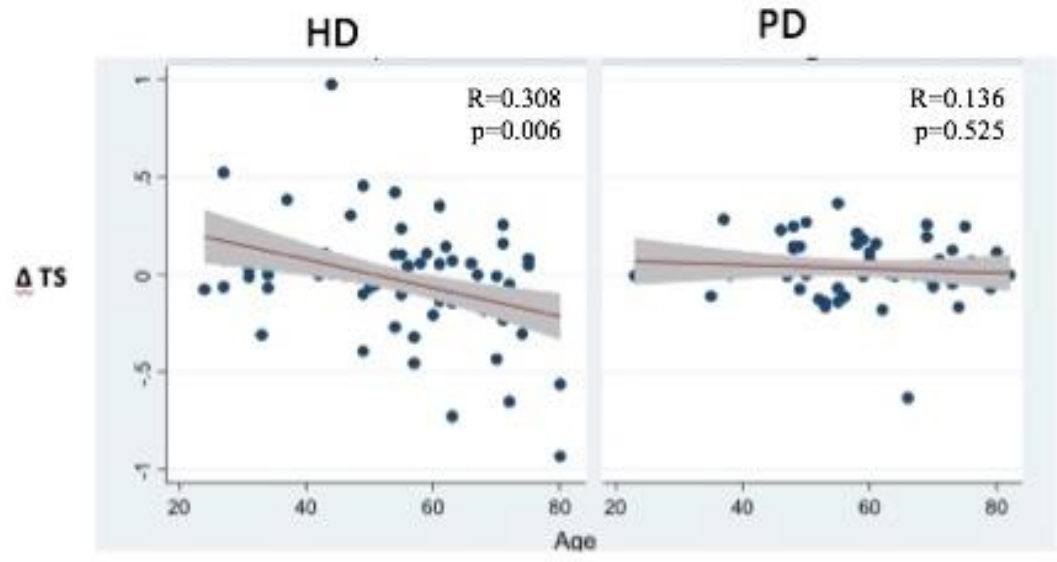

Figure 37: Correlation between delta TS and age in HD and PD group.

R=0.308 with p=0.006 in HD group. R=0.136 with p=0.525 in PD group.

Delta T/S	Estimate	Standard Error	95% Confidence Interval		P value
			Lower Bound	Upper Bound	
Female	0.0614	0.03880	-0.015	0.137	0.116
Age	0.0050	0.0014	0.002	0.008	**0.001**
PD	-0.0753	0.03801	-0.150	-0.001	0.050

Table 14: Linear regression model to analyse difference in delta TS between HD and PD group

Standardised T/S ratio	Coef.	Std. Err	95% confidence interval		P value
			Lower bound	Upper bound	
Age	-0.0108	0.0020	-0.0146	-0.0070	**0.000**
Female	0.1840	0.0545	0.0772	0.2907	**0.001**
Time	-0.0002	0.1395	-0.0384	0.0380	0.992

Table 15: Mixed model approach to estimate the change in T/S over time

6.3.6 The role of telomere length and frailty status among the dialysis cohort?

Data was available for 213 dialysis patients who had completed a frailty assessment at baseline as well as had their TL measured.

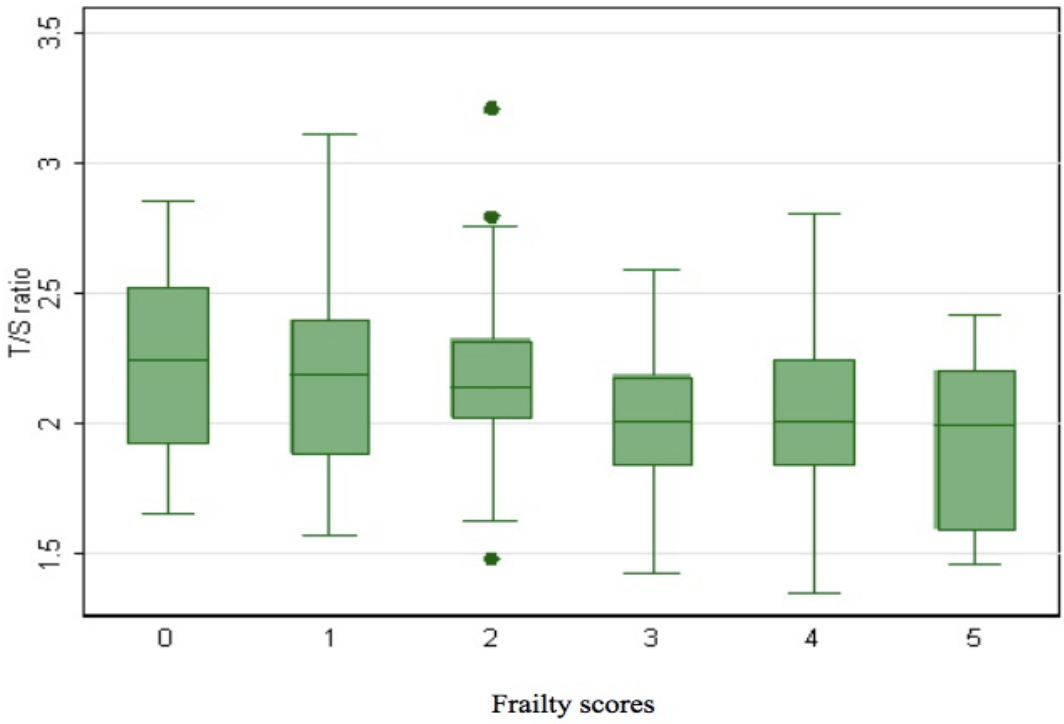

Figure 38: Frailty scores from 0 to 5 with corresponding T/S ratio values

A score of ≥ 3 from the frailty tool would indicate that the patient was frail whilst a score of ≤ 2 was considered non frail. Figure 38 shows the corresponding mean T/S ratio and distribution according to frailty status. The difference in mean T/S ratio was significant between frail and non-frail group p=0.001 (See Table 16).

Ageing parameters	Non frail (n=143)	Frail (n=70)	P value
T/S ratio (mean, SD)	2.17 (0.34)	2.00 (0.31)	**0.001**

Table 16: Univariate analysis of T/S ratio difference in non-frail and frail patients

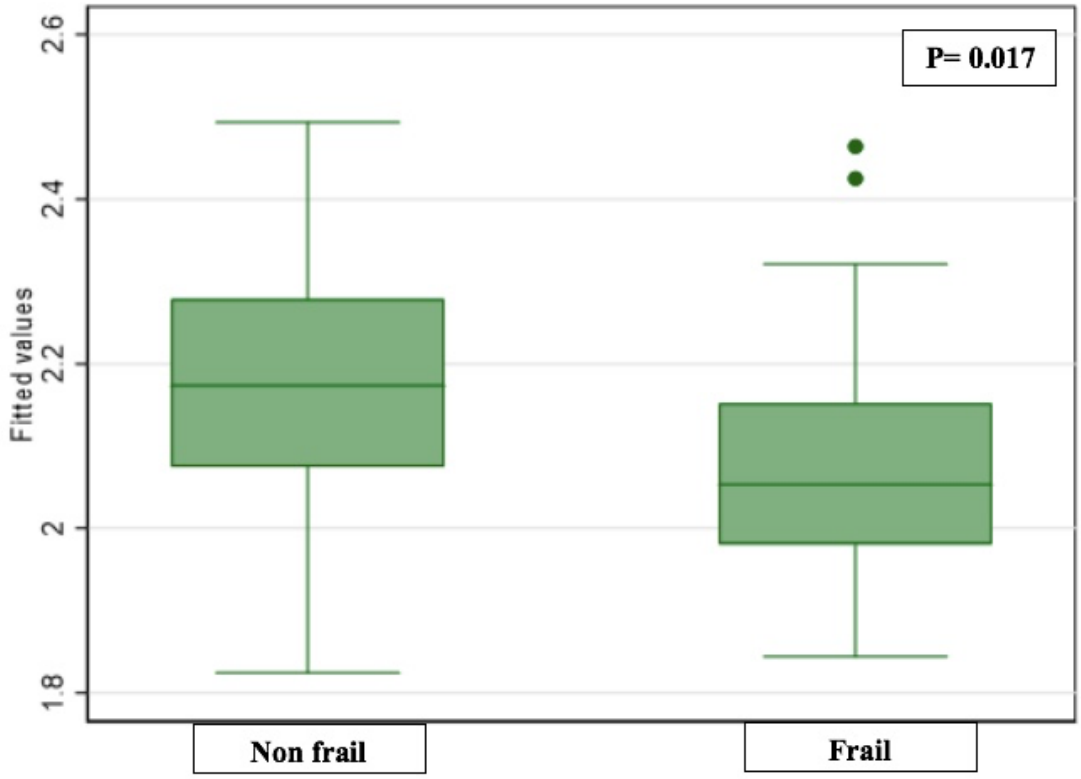

Figure 39: Adjusted TS ratio for age and sex. Non-frail and frail

T/S ratio	Beta	95 % Confidence Interval		P Value
		Upper bound	Lower bound	
Age	-0.00879	-0.01179	-0.00578	**<0.001**
Female	0.09376	0.00776	0.179759	**0.0328**
Frail	-0.1016	-0.1871	-0.0161	**0.0170**

Table 17: Linear regression model to compare frail vs non frail adjusting for age and sex

Frailty	Odds ratio	Standard Error	95% confidence interval		P values
			Lower Bound	Upper Bound	
Age	1.0303	0.0139	1.0047	1.0564	**0.020**
Female	0.7109	0.2326	0.37433	1.3500	0.079
ZTS	0.6567	0.1160	0.4646	0.9283	**0.017**

Table 18: Logistic regression model using standardised T/S ratio (ZT/S) with frailty as the outcome adjusted for age and sex

A logistic regression model was used to analyse the role of TL and frailty status in the dialysis cohort. The study showed a significant trend in frailty when patients were grouped into frail (those who score >=3) and non-frail group (those scoring <3) when regressed against TS ratio correcting for age and sex as potential confounders p= 0.017 (See Figure 39 and Table 17).

TL was standardised for this analysis to have a mean of 0 and SD of 1. Therefore, a unit increase in ZT/S will reflect a change in 1 SD. The risk of frailty is 48.8% higher for each SD decrease in T/S ratio after adjusting for age and sex in all patients and 52.2% higher for each SD decrease in the T/S ratio in the dialysis patients. However, this does not show that the rate of change in T/S ratio is different in dialysis patients and control group with regards to the risk of frailty. The data was modelled using all patients but adjusted for case to show how the risk of frailty due to TL was independent of dialysis. However, there are no frail controls found in this study. Therefore, the frailty status of all controls was discounted for the current analysis and further analysis. This does not reduce the impact of T/S ratio or cause any bias with the analysis because T/S ratio was not significant in both dialysis and control group adjusting for age and sex.

6.3.7 Is there an association between Frailty, Telomere Length and Dialysis Vintage

Frailty scores were available from patients with dialysis vintage of 2.14-1367.29 weeks. There was a significant correlation between frailty and dialysis vintage, R= 0.174, p<0.01. There was no correlation between dialysis vintage and T/S ratio in this study, R=0.093, p =0.470. The data was then analysed with a linear regression model by grouping dialysis vintage into quartiles (See Table 19).

Quartile	N	Minimum (weeks)	Maximum (weeks)
1	59	0	36.4
2	60	36.6	100.1
3	59	101.7	195.6
4	61	198	1367.3

Table 19: Quartile ranges of dialysis vintage in weeks of case groups

A multivariate analysis was conducted to assess the relationship between dialysis vintage in quartiles and T/S ratio following adjustment for age and gender (See Table 20). No significant association was found.

Z T/S	Coef.	Standard Error	95% confidence interval		P value
			Lower Bound	Upper Bound	
Age	-0.0281	0.0044	-0.0367	-0.0194	**0.000**
Female	0.0380	0.1277	0.0503	0.5534	**0.019**
Quartile 2	0.1978	0.1702	-0.1376	0.5333	0.246
Quartile 3	0.2384	0.1733	-0.1030	0.5798	0.170
Quartile 4	0.0805	0.1706	-0.0256	1.7189	0.637

Table 20: Multivariate analysis on effect of dialysis vintage and T/S ratio

Frailty	Odds Ratio	Standard error	95% confidence interval		P values
Age	1.025794	.0136932	.9993037	1.052986	0.056
Female	.6710864	.2337797	.3390422	1.328321	0.252
ZTS	.6494982	.1196786	.4526191	.9320153	**0.019**
TOD 2	2.646621	1.232402	1.062499	6.59257	**0.037**
TOD 3	1.047526	.5172768	.3979559	2.75737	0.925
TOD 4	3.04102	1.395366	1.23722	7.474661	**0.015**

Table 21: Logistic regression model to analyse the influence of dialysis vintage (time on dialysis =TOD) on standardised TS ratio (ZT/S)

The data was then adjusted to interrogate the Z T/S relationship with frailty but taking into account dialysis vintage as a probable confounder/risk factor for frailty. This was conducted to check that T/S ratio remained an independent predictor of frailty irrespective of time on dialysis. The odds of being frail were associated with longer dialysis vintage. The association between T/S ratio and frailty are independent of each other though there may be some confounding effect of dialysis vintage on this model. The effect of ZT/S and frailty (See Table 20 and Table 21) remained largely unchanged despite taking dialysis vintage into account.

6.4 DNA methylation analysis in dialysis patients

Data on DNA methylation was available in 228 dialysis patients. Missing data was observed in 11 patients (7 HD and 4 PD) due to loss of DNA integrity that led to a failure in the sequencing process. Even though all the samples were tested for DNA integrity, it may be that the subsequent repeated thawing process may have led to some degradation of the sample. Sequencing of DNA methylation status requires DNA with high structural integrity as outlined in the methodology section. As described in the methodology section, DNA methylation age is the predicted aged derived from the model built using information from 48 CpG sites. Apparent methylomic aging rate also better known as AMAR is the ratio of methylome derived predicted age to the true chronological age. Therefore, a higher value of AMAR means a higher predicted age which also means accelerated ageing. An AMAR >1 is synonymous with fast ageing and an AMAR <1 is synonymous with slow ageing (Kim and Jazwinski, 2015). DNA methylation delta age is the difference between the predicted age and chronological age. So, the higher the DNA methylation delta age, the higher the predicted or DNA methylation age is. Therefore, raised DNA methylation age represents accelerated ageing. The range of the methylation age observed in the cohort was between 18.54 years to 96.69 years with a mean of 56.17 ± 14.68.

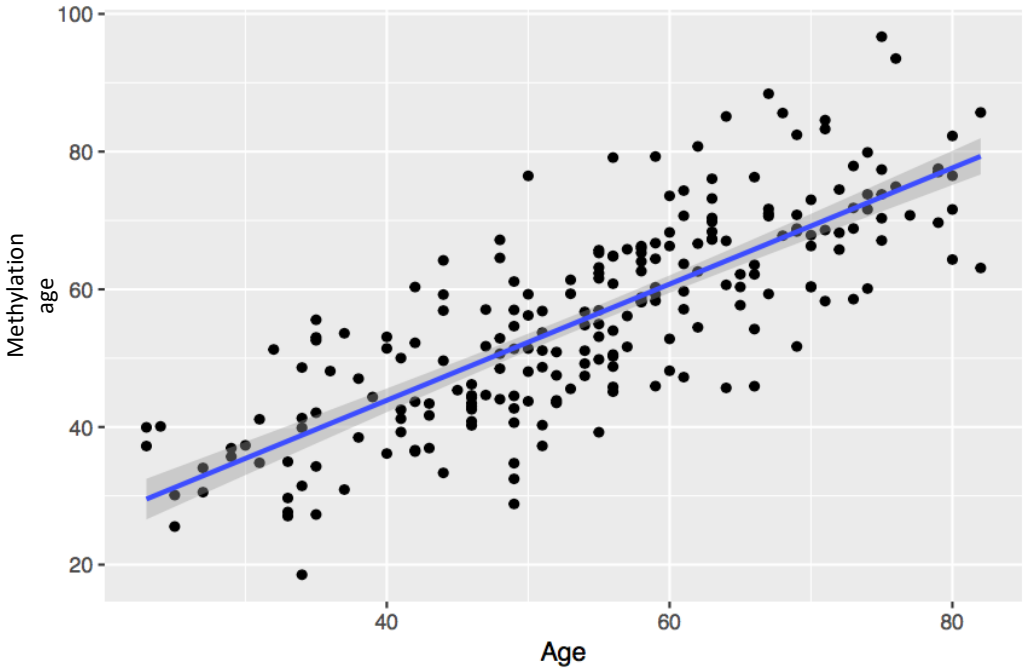

Figure 40: Correlation between DNA methylation age (predicted age) and age. R=0.79, p<0.001

Figure 40 shows a positive correlation between DNAm age and chronological age, R=0.79, p<0.001. Therefore, the older the individual the higher the DNAm age.

6.4.1 Relationship between DNA methylation and telomere length in the form of T/S ratio

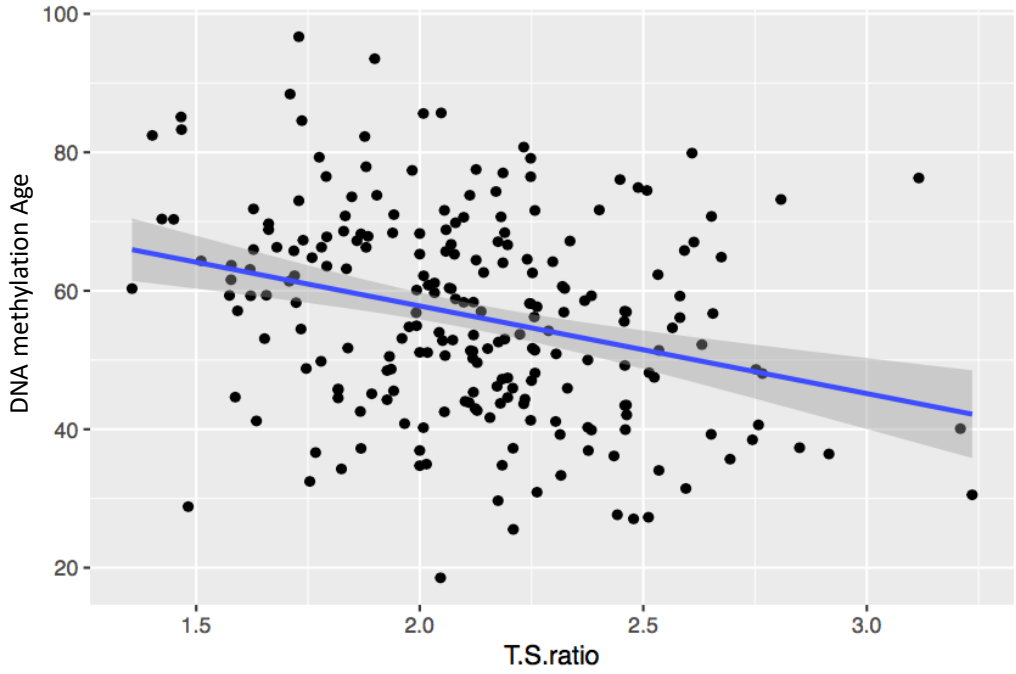

Figure 41: Correlation between DNA methylation age against T/S ratio. R= - 0.29, p<0.001

Figure 41 shows that a significant negative correlation between DNA methylation age and T/S ratio. This implies that the higher the predicted age the lower the T/S ratio consistent with the theory of ageing.

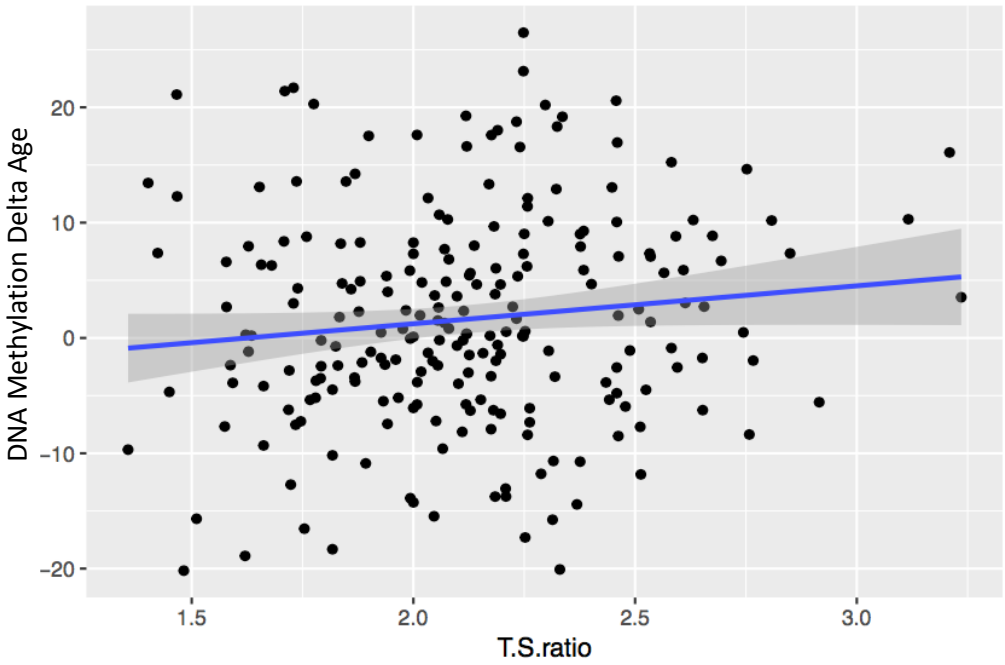

Figure 42: Correlation between DNA Methylation Delta Age against T/S ratio. R= 0.12, p=0.0712 (not significant)

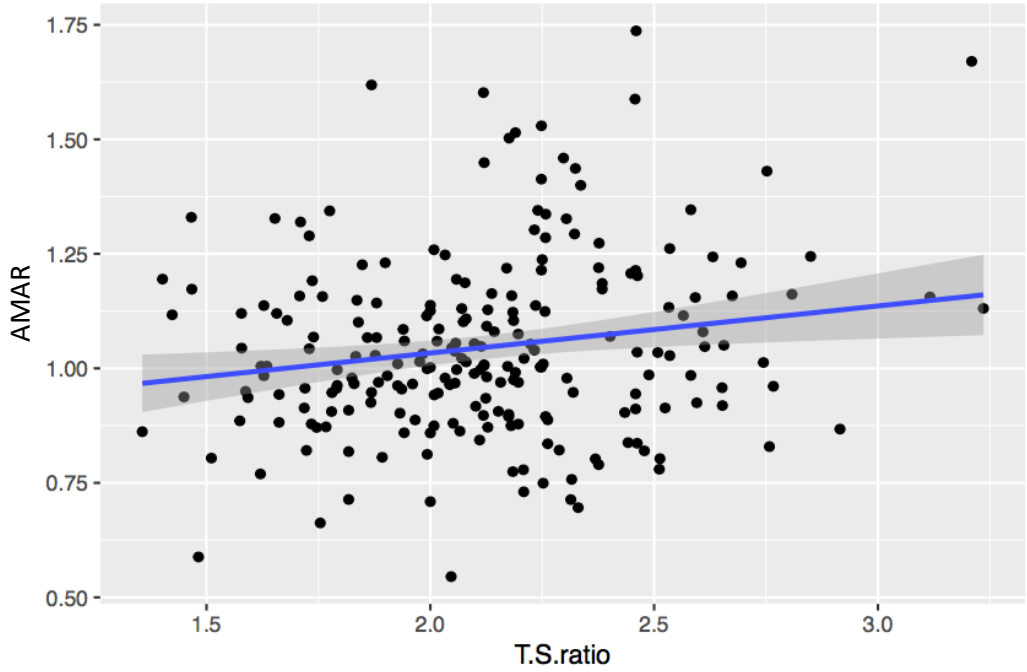

Figure 43: Correlation between AMAR against T/S ratio. R= 0.18, p=0.007

Figure 42 shows no significant correlation between DNA methylation delta age and T/S ratio. Figure 43 shows a significant correlation between AMAR and T/S ratio with an R =0.18. DNA methylation age had the best correlation with T/S ratio as seen in Figure 40. Table 22 shows the mean distribution for each DNAm ageing parameters and age among the 2 dialysis cohorts and their corresponding mean significance.

Ageing parameters	HD	PD	P values
Age (mean, SD)	53.24 (13.53)	55.65 (14.26)	0.181
DNA Methylation age (mean, SD)	55.35 (15.24)	57.06 (14.07)	0.381
DNA methylation delta age (mean, SD)	1.60 (8.96)	1.60 (9.60)	0.999
AMAR (mean, SD)	1.04 (0.19)	1.05 (0.21)	0.634

Table 22: Chronological age and Methylation ageing parameter difference between HD and PD.

6.4.2 Role of DNA methylation markers in frailty among the dialysis cohort?

The study was aimed at investigating if biomarkers of ageing can predict frailty in the dialysis cohort. Results on frailty have been extensively discussed in Section 6.2.

Ageing parameters	Non frail (n=143)	Frail (n=70)	P
Methylome age (mean, SD)	54.28 (14.60)	61.41 (13.66)	**0.001**
DNA m delta age (mean, SD)	1.75 (9.57)	2.22 (8.96)	0.736
AMAR (mean, SD)	1.05 (0.21)	1.05 (0.17)	0.947

Table 23: Baseline comparison of ageing parameters between frail and non-frail group

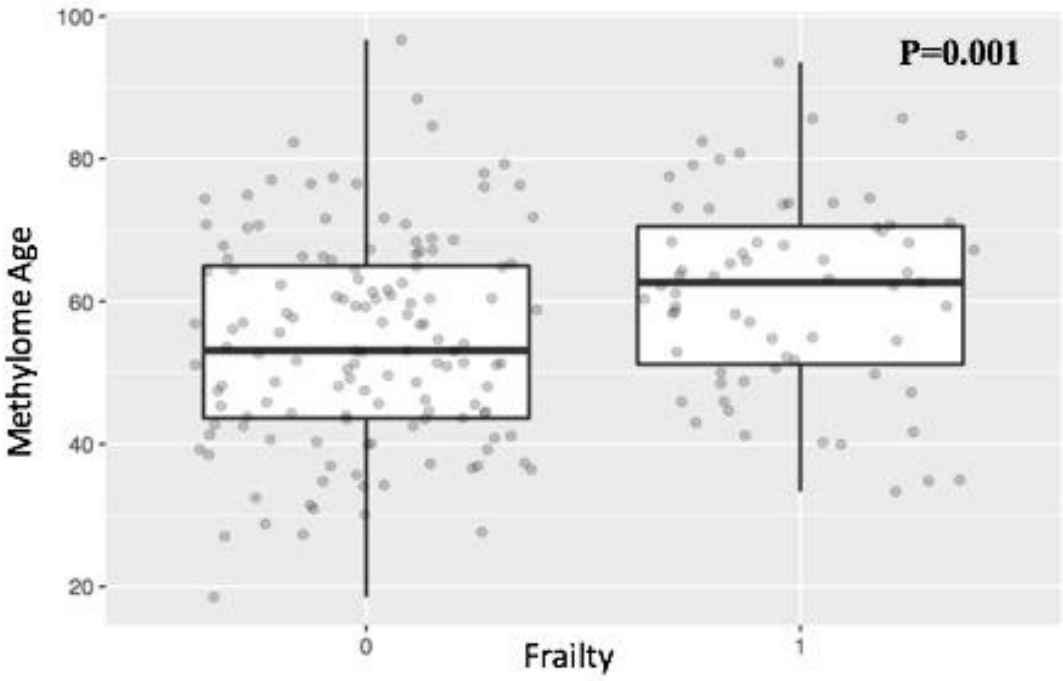

Figure 44: Box plot showing the distribution and spread of methylome age against frailty. 0 is not frail and 1 is frail.

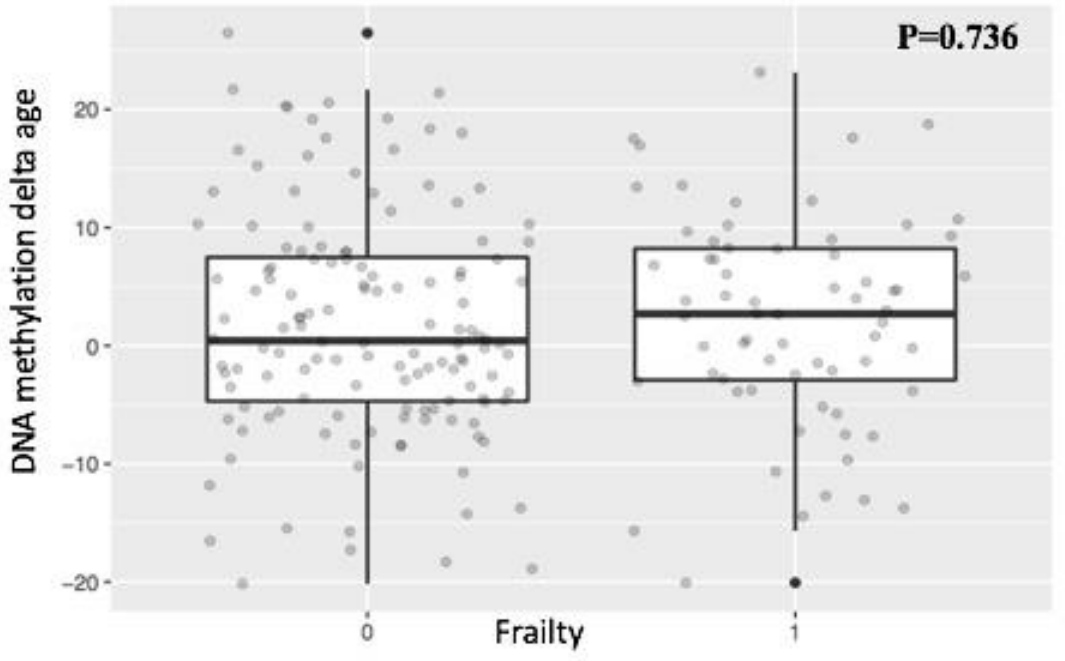

Figure 45: Box plot showing the distribution and spread of DNA m delta age (methylome age minus chronological age) against frailty. 0 is not frail and 1 is frail

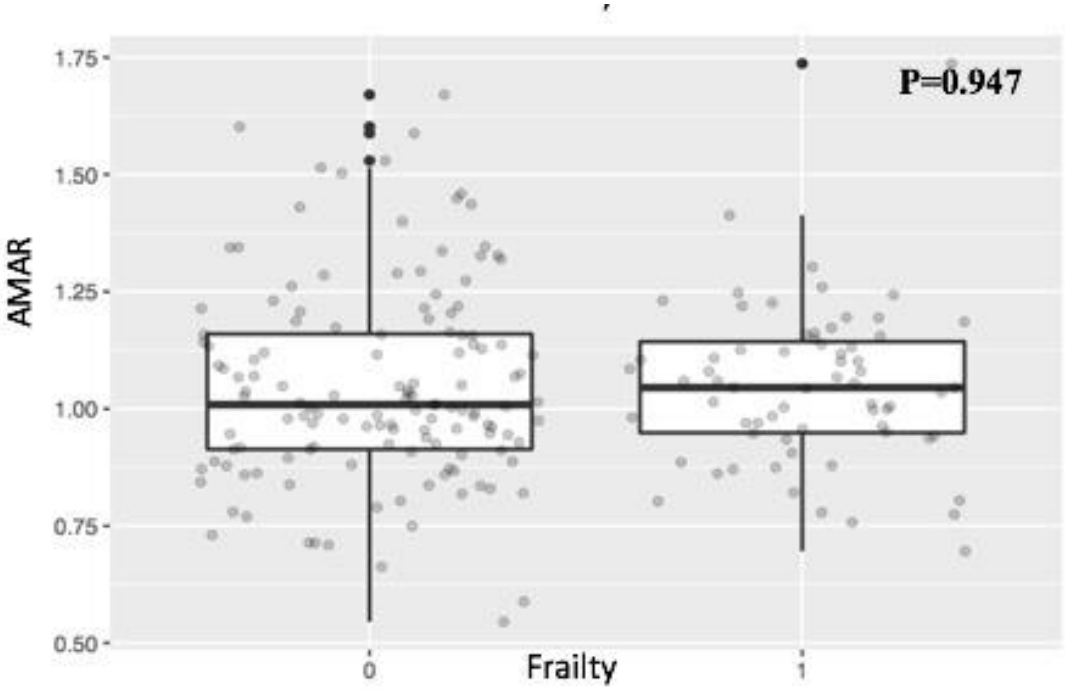

Figure 46: Box plot showing the distribution and spread of AMAR (methylome age/chronological age) against frailty. 0 is not frail and 1 is frail

Figure 44, 45 and 46 are box plots looking at the spread of methylation markers in both frailty groups. Increased methylation age was observed in the frail group in comparison to the non-frail group, this was significant, p<0.001. However, a multivariate analysis performed with frailty as the outcome and methylation age as the predictor adjusted for age and gender revealed no significance, p=0.245. An increase in mean methylation age by 0.02 years is associated with being frail after adjusting for age and sex.

Frailty	Odds ratio	Standard Error	95 % confidence interval		P value
			Lower Bound	Upper Bound	
Methylome age	1.020	0.017	0.986	1.055	0.245
Age	1.022	0.019	0.985	1.061	0.246
Female	0.678	0.332	0.353	1.300	0.242

Table 24: Multivariate analysis of methylation age as predictor and frailty as outcomes.

Frailty	Odds ratio	Standard Error	95% Confidence Interval		P value
DNAm delta age	1.020	0.017	0.986	1.055	0.245
Age	1.043	0.013	1.018	1.069	**0.001**
Female	0.678	0.332	0.353	1.300	0.242

Table 25: Multivariate analysis of DNAm delta age and frailty outcomes

Frailty	Odds ratio	Standard Error	95% confidence interval		P value
AMAR	2.777	0.887	0.488	15.787	0.249
Age	1.044	0.013	1.018	1.071	**0.001**
Female	0.669	0.334	0.348	1.288	0.229

Table 26: Multivariate analysis of AMAR and frailty outcome

Table 25 and 26 show that chronological age is significantly associated with DNA methylation age, DNAm delta age and AMAR. This is because DNAm delta age and AMAR are calculated based on chronological age. DNA methylation age-based model is built from chronological age of healthy adults which will account for the high correlation between these parameters.

6.5 Predictors of frailty in the dialysis cohort

Variables	Odds ratio	Standard Error	95% Confidence Interval		P values
			Lower Bound	Upper Bound	
Age	1.0391	0.0117	1.0155	1.0632	**0.0011**
Female	0.6466	0.3118	0.3510	1.1913	0.1620
Asians	1.2755	0.3315	0.6660	2.4426	0.4630
Blacks	0.7623	0.3991	0.3487	1.6667	0.4966
CCI score 3	11.958	0.4812	4.6564	30.7094	**0.000**
CCI score 4	4.5455	0.4536	1.8686	11.0573	**0.000**
CCI score 5	14.546	0.5250	5.1981	40.7013	**0.000**
CCI score >5	18.409	0.6805	4.8502	69.872	**0.000**
BMI	0.9940	0.0282	0.9407	1.0504	0.8313
Smoking current	1.35	0.4517	0.5570	3.2719	0.5064
Ex-smokers	0.8617	0.3296	0.4517	1.6440	0.6515
Haemoglobin	1.2141	0.0984	1.0012	1.4722	**0.0486**
WCC	1.1909	0.0700	1.0382	1.3661	**0.0126**
Albumin	0.8725	0.0392	0.8080	0.9421	**0.0005**
CRP $\geq$ 5	2.5578	0.2990	1.4236	4.5957	**0.0017**
Modality PD	0.4630	0.3007	0.2568	0.8347	**0.0104**

Table 27: Univariate logistic regression to predict frailty in the dialysis cohort.

Table 27 above shows that age, Charlson Comorbidity index of ≥ 4, Haemoglobin, White cell count, Albumin and C-reactive protein, modality have significant associations with frailty as an outcome.

Variables	Odds ratios	Standard Error	95% Confidence Interval		P-value
			Lower Bound	Upper Bound	
Age	1.0391	0.0117	1.0155	1.0632	**0.0011**
T/S ratio	0.2082	0.4780	0.0816	0.5313	**0.0010**
Methylome Age	1.0355	0.0109	1.0135	1.0579	**0.0014**
DNAm delta age	1.0054	0.0160	0.9744	1.0375	0.7347
AMAR	0.9506	0.7568	0.2157	4.1896	0.9467

Table 28: Univariate logistic regression on biomarkers of ageing to predict frailty including age

Table 28 highlights that both T/S ratio and methylome age have significant association with frailty as an outcome in a univariate logistic regression despite the fact that both T/S ratio and methylome age had a weak correlation, R=0.29 (See Figure 41).

All the significant variables from the univariate logistic model as seen in Table 27 and 28 in predicting frailty as an outcome were selected to fit a multivariable logistic regression model.

Variable	Odds ratios	Standard error	95% Confidence Interval		P-value
			Lower Bound	Upper Bound	
T/S ratio	0.211	0.693	0.054	0.820	**0.025**
Age	0.980	0.027	0.930	1.033	0.449
Modality PD	0.167	0.481	0.065	0.429	**0.000**
CCI score 3	16.364	0.620	4.851	55.20	**0.000**
CCI score 4	7.538	0.594	2.352	24.16	**0.001**
CCI score 5	19.457	0.723	4.720	80.20	**0.000**
CCI score >5	22.814	0.806	4.699	110.75	**0.000**
Hb	1.418	0.147	1.062	1.892	**0.018**
WCC	1.179	0.096	0.977	1.423	0.086
Albumin	0.861	0.067	0.756	0.981	**0.025**
CRP ≥ 5	2.636	0.419	1.158	5.997	**0.021**
Methylome age	1.007	0.022	0.964	1.052	0.759

Table 29: Multivariable logistic regression model to predict frailty as an outcome for T/S ratio. Methylome age was found to have no significance in the multivariate model, p=0.968.

A multivariate analysis was repeated for methylome age instead of T/S ratio to assess if there was a change in significance (See Table 30).

Variable	Odds ratio	Standard Error	95% Confidence Interval		P value
			Lower Bound	Upper Bound	
Methylome age	1.006	0.015	0.977	1.035	0.694
PD	0.214	0.445	0.089	0.511	**0.001**
CCI 3	15.464	0.587	4.893	48.870	**0.000**
CCI 4	6.606	0.567	2.176	20.058	**0.001**
CCI 5	22.432	0.685	5.864	85.810	**0.000**
CCI >5	25.416	0.784	5.468	118.143	**0.000**
Hb	1.383	0.138	1.055	1.813	**0.019**
Alb	0.891	0.062	0.789	1.007	0.065
CRP ≥5	2.795	0.409	1.254	6.230	0.012

Table 30: Multivariate logistic regression for methylome age to predict frailty as outcome. The table only reports significant variables.

Table 28 highlights that there was no change in significance of methylome age in predicting frailty as an outcome in a multivariate logistic regression which was performed without T/S ratio.

6.5.1 Receiver Operating Characteristic curve analysis

Receiver operating characteristic (ROC) curve analysis was performed for T/S ratio and DNA methylome age against the development of frailty. The graphical plot determines the diagnostic ability of a variable to predict a binary outcome (e.g. frail Vs non-frail). Using every value of the variable as a cut-off point, the sensitivity (true positive rate) of a variable is plotted against the corresponding 1-specificity (false positive rate). The greater the area under the resulting curve, the more useful the test is at predicting the outcome. An area under the curve (AUC) of 1 represents a test that predicts the correct outcome by a 100% whilst an AUC of 0.5 implies that the measurement of interest was unable to distinguish the outcome. Generally, a positive test outcome requires an AUC of >0.5.

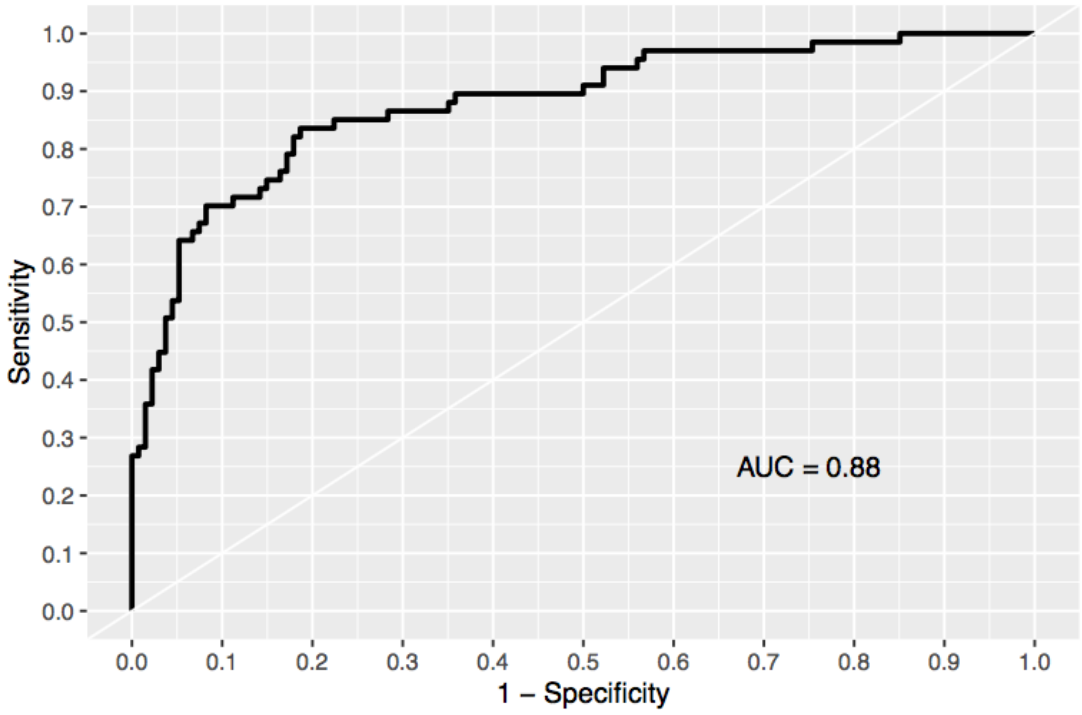

Figure 47: A ROC curve analysis showing the predictive ability of the significant variables from the multivariate logistic regression with AUC =0.88

The Receiver operator characteristics (ROC) curve was built taking into account all the significant variables from the multivariate logistic regression analysis from Table 29 to measure the predictive ability of these factors. A combination of these variables produced an AUC of 0.88. Table 29 shows that both methylome age and age becomes insignificant with frailty as outcome, therefore were not included in the ROC curve analysis.

There were 9 significant variables from the multivariate logistic regression analysis which produced an AUC of 0.88 to predict frailty. However, these results may be affected by 'overfitting of the model'. This is because there were only 70 events in the form of frail patients and by applying the 'rule of 10' would mean that each variable tested will have an average outcome/contribute to 10 events. So, this would imply that the model had to be adjusted to include only 7 variables. This was achieved by further categorising Charlson's Comorbidity Index to two different levels. A multivariate logistic regression analysis was repeated with the new group of variables to generate a ROC curve (See Table 31 and Figure 48).

Variables	Odds ratios	Standard Error	95% Confidence Interval		P values
			Lower Bound	Upper Bound	
T/S ratio	0.194	0.673	0.052	0.725	**0.015**
Age	0.982	0.026	0.932	1.034	0.481
PD	0.171	0.472	0.068	0.432	**0.000**
CCI >2	13.103	0.508	4.843	35.45	**0.000**
Hb	1.463	0.142	1.107	1.934	**0.007**
WCC	1.194	0.094	0.993	1.434	0.059
Alb	0.857	0.065	0.754	0.974	**0.018**
CRP ≥ 5	2.630	0.408	1.182	5.849	**0.018**
Methylome age	1.010	0.022	0.967	1.055	0.648

Table 31: Multivariate logistic regression analysis for T/S ratio

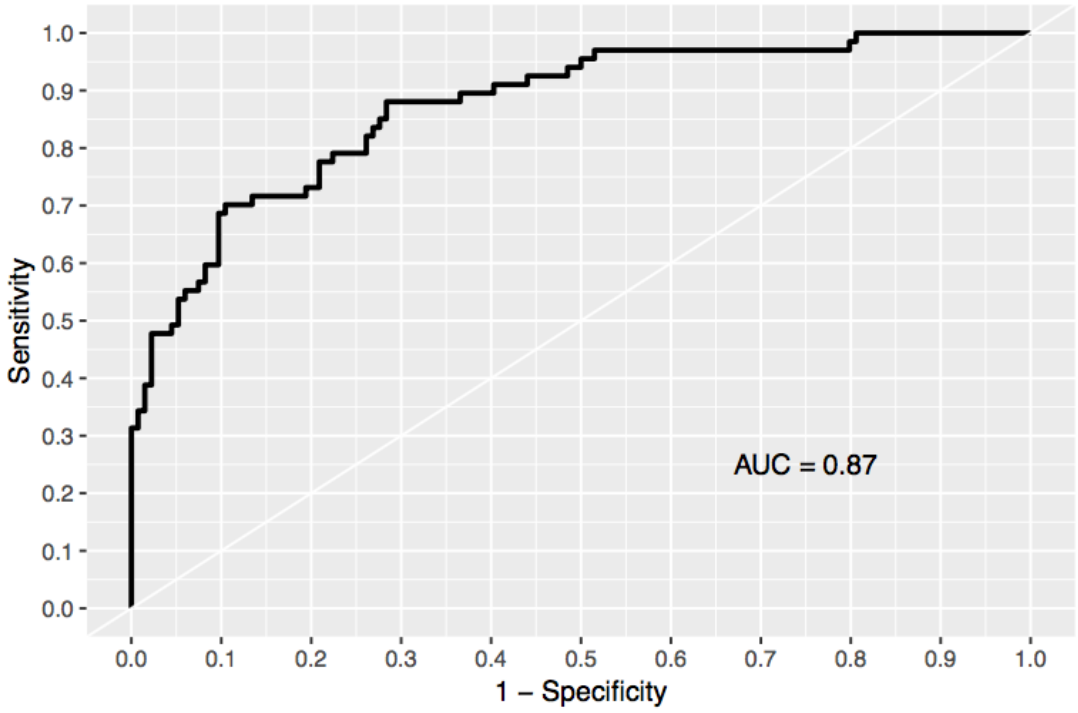

Figure 48: ROC curve analysis to predict frailty with significant variables from multivariate logistic regression analysis

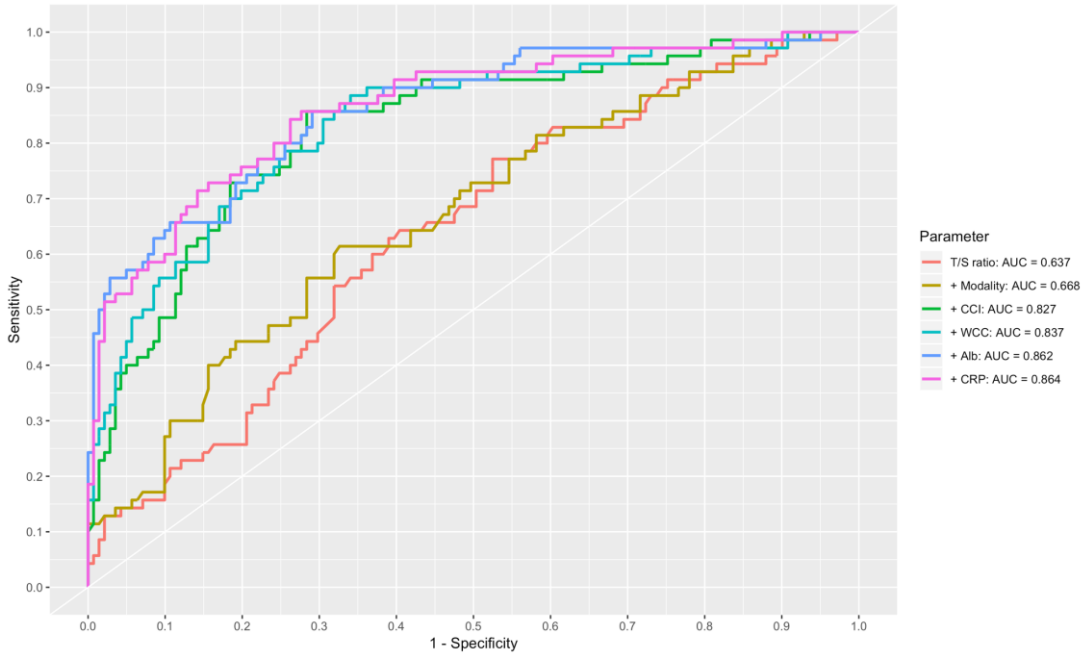

Figure 49: A ROC plot adding the adjusted covariates one by one, but not including the insignificant variables. The legend outlines the order in which the variables were added to the ROC plot.

The ROC curve above is a step wise ROC curve analysis which was done to investigate the effect of each variable and changes to the AUC. Adjusted covariates that were significant from the multivariate analysis were added one by one to see the progressive change in the AUC. From the ROC plot, it appears that the addition of Charlson's Comorbidity Index significantly changes the AUC from 0.668 to 0.827, meaning that CCI has a significant role in predicting frailty.

6.5.2 Biomarkers of ageing as predictors of frailty status in dialysis patients (Univariate Logistic Regression Analysis)

A ROC curve was also built using the three different measures of ageing which are shown in Figure 50 with corresponding AUC values. Chronological age, methylome age and T/S ratio were statistically significant predictors of frailty in the dialysis cohort. Of the three, T/S ratio was most significantly associated with predicting frailty with AUC of 0.637, $p<0.001$. DNA methylation delta age and AMAR were not included in the ROC curve analysis due its dependence on age and these variables were not significant from the univariate logistic regression with frailty as outcome (See Table 32).

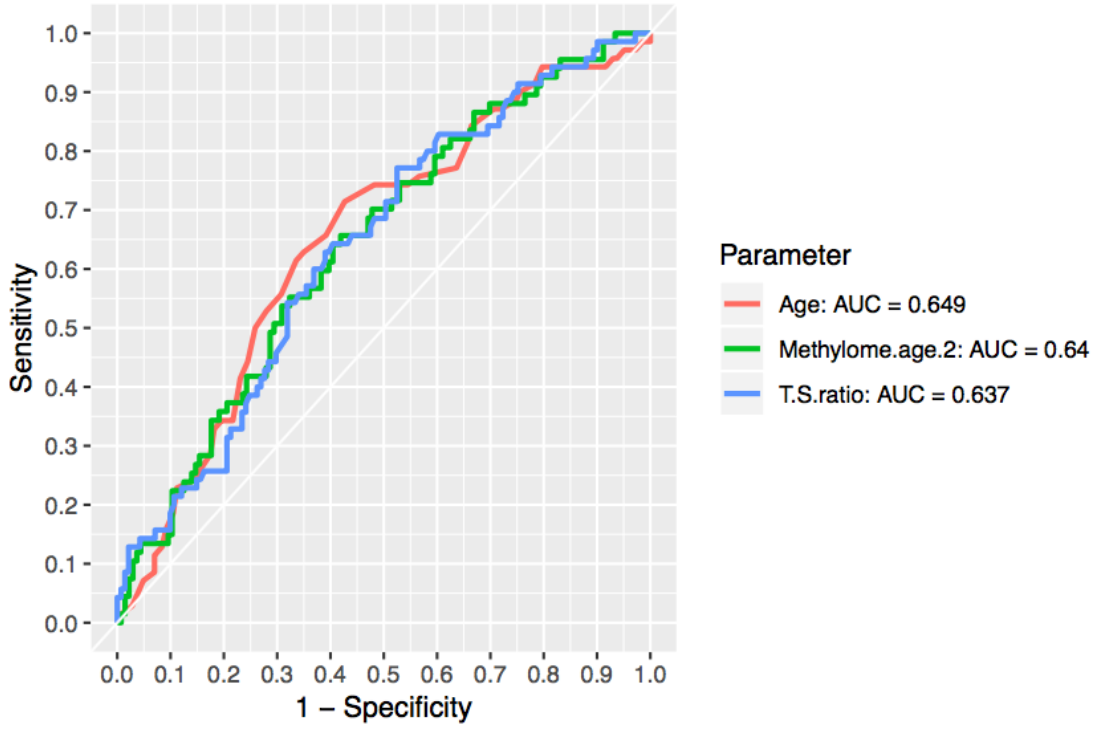

Figure 50: ROC curve demonstrating the diagnostic ability for the three measured ageing parameters in predicting frailty in the dialysis cohort.

Test Result Variable	Area Under Curve	Standard Error	95% confidence interval		P-value
			Lower Bound	Upper Bound	
Age	0.6492	0.0117	0.5709	0.7275	**0.0011**
T/S ratio	0.6375	0.4780	0.5596	0.7154	**0.0010**
DNAm Age	0.6404	0.0109	0.5608	0.7200	**0.0014**
DNAm delta age	0.5291	0.0160	0.4451	0.6130	0.7347
AMAR	0.5192	0.7568	0.4373	0.6011	0.9467

Table 32: Diagnostic ability of chronological age, T/S ratio, DNA methylome age, DNA methylation delta age and AMAR for predicting frailty. P values for statistically significant predictors are in bold.

CHAPTER 7:DISCUSSION

7.1: General Discussion

The process of ageing remains to be a complex phenomenon that is heightened in uraemia and remains a challenge; not only when it comes to understanding the factors involved but also when identifying methods to measure ageing in uraemia. As described in Chapter 1, the aim of the PhD was to gain a better insight in assessing and quantifying premature ageing in uraemic patients through parameters that have shown promise in healthy control and non-CKD patients.

As elaborated in Chapter 1, TL is a well-established novel biomarker of ageing that declines with increasing age and has been associated with survival outcomes in healthy donors. Since the discovery of this biomarker of ageing that represents biological age, there has been a surge in research and publications in the field of cardiovascular and cancer and ageing. However, only a handful of researchers have investigated the role of telomere biology as an objective ageing marker in uraemia. Another biomarker of ageing that has gained recognition is frailty; a phenotypic measure that represents overall health and functional status which has a great impact on survival. Frailty has been studied among patients with uraemia through various screening tools, but no consensus has been established on the criteria used to define frailty in these patients. There is also limited data on the use of frailty phenotype as a subjective ageing biomarker in uraemia.

The parameters that were studied to fulfil the aim of this Ph.D. was the role of TL and frailty phenotype as markers representing ageing in uraemia. This was the first study to investigate if there was any association between TL, DNA methylation and frailty phenotype as biomarkers of ageing in patients with end stage kidney disease. Results of these outcomes of interest will be discussed below.

7.2 Discussion on frailty outcomes in study cohort

7.2.1 Age and gender effect on frailty status in dialysis patients

There was no significance between frailty status and gender found among 212 patients in this study. In an older population, the prevalence of frailty was higher in women than men. (Collard *et al.*, 2012). Similar findings were also seen in patients on dialysis from the Dialysis Morbidity and mortality study (DMMS) Wave 2 consisting of 3931 patients from HD and PD whereby women were more likely than men to be frail in all categories of age. However, ACTIVE/ADIPOSE (A Cohort Study to Investigate the Value of Exercise in ESKD/ Analyses Designed to Investigate the Paradox of Obesity and Survival in EKRD); a multicentre study of 745 patients on HD who had their frailty status measured revealed no association between female sex and frailty (Kutner, 2014). Similar findings were also noted by a recent study conducted by McAdams-DeMarco et.al (McAdams-Demarco *et al.*, 2013). These 2 studies had used performance-based measures for muscle strength and gait speed. The DMMS Wave 2 study by Johansen et.al and Bao et.al both showed than women were frailer than men and had utilised patient reported physical function questionnaires as a substitute for

grip strength and walk assessment. A patient reported health status measure representing physical function is clearly a different metric from a performance based assessment of muscle strength and gait speed which could account for the different outcomes seen with regards to gender and frailty (Painter and Kuskowski, 2013)(Kutner, 2013). The baseline data from the ACTIVE/ADIPOSE study revealed that 61% of patients scored less than 75 based on the SF-36 PF scale but only 5% were found to be frail in both measured grip strength and walk speed (Kutner, 2013). These findings underscore the potential for misclassifying patients as frail when using a self-reported physical function questionnaire instead of a performance-based assessment of weakness and slowness domain.

The sample size of this study was limited but consisted of 212 patients from 2 different types of dialysis modality having completed their frailty assessments at baseline. Even though no association was seen between gender and frailty as observed by other larger studies it is important to note that the gender distribution in this study cohort was unequal and females were younger than men. The statistics in this study was corrected for age and gender whenever possible.

The prevalence of frailty increases with age in the general population (Hanlon *et al.*, 2018). Frailty has generally thought to be a phenotype predominant in the elderly however among uraemic patients on dialysis, frailty occurs at an early age and frequently below the age of 65. Our work demonstrated a significant correlation between age and frailty scores. Frailty was seen in 12% of patients under the age of 40 year and 26% between the ages of 40-50 years from a total of 212 patients. This is in similar to data from DMMS Wave 2 study showed that 44% of patients under the

age of 40 and more than 50% of patients between the ages of 40-50 were frail out of a total of 2393 patients. In non-dialysis patients, older age was clearly associated with frailty but data from DMMS Wave 2 study also showed that there was lack of interaction between age and frailty suggesting that a significant proportion of younger dialysis patients were also frail (Johansen *et al.*, 2007). Frailty in patients under the age of 40 years from the Comprehensive Dialysis Study consisting of 1576 patients revealed that 63% were frail (Bao *et al.*, 2012). Taken together it is clear that ESKD is a strong risk factor for frailty particularly in the young. While our current study demonstrates significant rates of fragility in young dialysis population, the other larger studies found an even higher prevalence of frailty. Possible explanations for this may be due to a difference in different scoring systems in comparison to the one used in the current study that could explain the differences observed. A modified version of the Fried criteria for frailty were applied in these large studies to provide a final score on frailty that did not utilise hand grip strength to assess weakness but instead used the Rand 36 PF and SF 12 PF questionnaire to provide a score for this component which may have led to a relative overestimation of frailty in the larger cohort studies.

7.2.2 Frailty phenotype among haemodialysis and peritoneal dialysis patients

The prevalence of frailty is 6.9% in community dwelling older adults (Fried *et al.*, 2001) and thought to be much higher in patients with ESKD. The current data suggests that the prevalence of frailty among dialysis cohort ranges from 3 to 10 fold higher than community-dwelling elderly (Nitta, Hanafusa and Tsuchiya, 2017). The Fried

Frailty Phenotype is commonly applied among dialysis patients however the prevalence was found to be lower when measured by this method but remained to be significantly much higher by 4 to 6 times in comparison to healthy elderly adults (Nitta, Hanafusa and Tsuchiya, 2017). There has been variability between frailty status between ESKD cohorts probably a result of different methods used to assess frailty.

This study demonstrates that among 70 frail patients in total, 64.3% were on haemodialysis at the time of recruitment. Among the whole study cohort consisting of 213 patients, frailty was observed in 21% of haemodialysis and 11.8% peritoneal dialysis patients despite the mean age of PD cohort and female distribution being similar in HD cohort. This study showed that the overall prevalence of frailty phenotype in patients on HD was substantially higher (21%) in comparison to community-dwelling older adults (6.9% in the Cardiovascular Health Study and 16.3% in the Women's Health Initiative Observational Study) (Fried *et al.*, 2001)(Woods *et al.*, 2005). The DMMW 2 study showed that HD rather than PD modality was independently associated with frailty with an odds ratio of 0.80 (95% CI 0.65-0.97) (Johansen *et al.*, 2007). However, a large study consisting of 1616 Korean patients comparing 1250 patients on HD and 366 patients on PD revealed the opposite finding (Kang *et al.*, 2017)(Lee *et al.*, 2017). There was no significant difference in frailty between patients treated with the 2-different dialysis modality in the Korean studies. The dialysis vintage in the Korean studies aforementioned were similar in HD and PD. The DMMW 2 study had no information on dialysis vintage. The studies by Johansen et.al., Kang et.al and Lee et.al. had utilised the questions from KDQOL and RAND 36 and SF 36 which both consist of identical items. The possibility for a reduction in the frequency of frailty observed in the PD cohort in comparison to the

HD cohort could be explained by a shorter dialysis vintage in our study. Another important factor is that patients are not randomly assigned to PD vs HD: in our pre dialysis clinic the patient's functional status is considered when counselled for treatment modality. A shared decision is made with the patient, with PD recommended for those patients who are more self-caring, ambulant and have less cognitive impairment. This may also account for HD patients having a higher frailty score in comparison to PD patients. There are also a small number of patients on assisted PD due to impaired functional status, but this study did not have a record of patients on assisted PD.

7.2.3 Dialysis vintage and frailty status in dialysis cohort

There was strong correlation seen between dialysis vintage and frailty status among our cohort. Exposure to prolonged periods of dialysis is associated with various adverse outcomes which subsequently affect ones' frailty status directly and indirectly. Increased dialysis vintage is associated with accumulated losses of nutrients during dialysis/protein energy wasting (PEW), inflammation as a result of oxidative and carbonyl stress, superimposed catabolic illness and stress from the dialysis procedure, prolonged exposure to uraemic toxins and altered hormone profiles; factors that contribute to frailty status measured by the 5 domains of Fried Frailty Phenotype.

Duration of dialysis is a relative contributor towards frailty and ageing in patients with ESKD (Kim, Kalantar-Zadeh and Kopple, 2013). A retrospective cohort study of 216 246 patients receiving maintenance HD for more than a year revealed that longer

dialysis vintage was associated with lower level of body mass index (BMI) which reflects loss of muscle mass (Sumida *et al.*, 2016). An cross-sectional study showed that 31.5% of patients have sarcopenia as defined by the European Working Group on Sarcopenia in Older People (EWGSOP) which was associated with a longer dialysis vintage (Bataille *et al.*, 2017). Loss of muscle mass or sarcopenia is associated with physical inactivity and reduced hand grip strength which would affect the frailty domains of exhaustion, weakness, slowness, physical activity and weight loss. In fact, the frailty scores of the dialysis cohort in this study are more likely to be higher as weight loss was reported by patients based on their reflection of recorded weight measured a year ago. Patients may not be aware of age-related changes in body composition causing sarcopenic obesity which refers to a reduction in muscle mass but matched by the gain in body fat (Gallagher *et al.*, 2000).

A study of 97 elderly patients with a mean age of 84.5 years revealed that 78% were independent in their activities of daily living (ADL) at the time of dialysis initiation, but >30% of patients had functional loss after 6 months of dialysis treatment and their frailty level, defined by loss of independence continued to decline during a 2.5 year follow up period (Jassal, Chiu and Hladunewich, 2009). US registry data from nursing home residents with a mean age of 73.4 ± 10.9 revealed a downward trajectory in their functional status assessed from 7 ADL from 3 months prior to dialysis initiation to 12 months of dialysis treatment (Kurella Tamura *et al.*, 2009). Even though these studies did not formally measure frailty using the Fried criteria, the clinical outcomes do support a decline in functional status over a period of time on dialysis. However, the patients included in these studies were much older and data from the national

registry in US were in nursing home patients who are likely to have a high prevalence of disability at baseline to start off with.

There have been few studies which have reported outcomes on frailty scores and dialysis vintage. The ACTIVE-ADIPOSE study failed to show any association between length of time on HD and frailty status with a median dialysis vintage of 3.3 years (Kutner, 2014). A single prospective study of haemodialysis cohort of 146 patients did not reveal any association between frailty status and time on HD (McAdams-Demarco *et al.*, 2013). In a prospective study of 1658 patients (1255 on HD and 403 on PD) showed that frailty was not related to the dialysis duration of 5.2 ± 4.5 years (p= 0.744), however this study used the Korean version of Kidney Disease Quality of Life SF36 questionnaire to measure frailty; a modified version of the Physical Frailty Phenotype whereby all the frailty components were assessed from questionnaires with no objective measures (Lee *et al.*, 2017). The studies had no information on frailty status prior to HD initiation or longitudinal data which may have affected the outcome of frailty status and dialysis vintage.

7.2.4 Changes in frailty status over time

This study was a 1-year longitudinal study aiming to ascertain if there was any significant change in frailty status from baseline. There was no significant difference seen between overall frailty score at baseline and after 12 months in the dialysis group and there was no difference between HD or PD group. This is because the change in frailty score of 5 components was too small to establish any significant difference in

a year and perhaps the change in frailty occurs over a longer period of time. The study also excluded any patient with a life expectancy of 6 months or less which reflects the frailest group on dialysis which would have inevitably affected the overall frailty scores and 1 year follow up scores if these patients survived. It is difficult to know if the largest difference in frailty is seen before initiation of dialysis and a specific time frame post dialysis as there are no studies that have looked into this aspect. There were 69 patients who had transitioned over to transplantation. A subgroup analysis of this cohort revealed that there was a significant difference in exhaustion at baseline and 1-year post transplant. The significance of this finding may be affected as the protocol for the study did not censor or exclude patients who had transitioned into transplantation. Therefore, the duration of these patients having a renal transplant varied from 2 months to 12 months. Studies evaluating frailty post transplantation has been well published in the literature but there are no longitudinal studies to assess frailty pre and post transplantation. These findings are certainly worth investigating further especially since recent data revealing that telomere attrition is more pronounced post renal transplantation than on dialysis (Luttropp *et al.*, 2016).

Frailty is bidirectional and potentially reversible. There has been consistent evidence on the positive effects of aerobic exercise on physical fitness, muscular strength and quality of life in ESKD patients (Jhamb *et al.*, 2008). So, there needs to be greater emphasis in applying these measures or interventions to address modifiable aspects of frailty components in the dialysis population at all health institutions. This can include working together with primary care to providing aerobic and anaerobic exercise regimes which can be administered in the dialysis unit or in the community. There are cycling apparatus available at dialysis units for the use of patients and improving

access to this by incorporating an exercise regime to treatment will be step forward in the correct direction to address physical inactivity in dialysis patients. There needs to be greater emphasis on nutrition and education not only to patients but especially for those caring for ESKD individuals. Perhaps a cooking video providing nutrition ideas and recipes for dialysis patients to incorporate cultural values which can be easily accessible from the trust website. Clearly this will need to be tailored according to units due to variation in patient population and ethnicity.

There is also need for longitudinal studies to assess frailty status over a longer period of time to investigate practical and cost effective intervention strategies which may lead to promising findings as frailty is reversible as a duration of 1 year may not be sufficient enough to see a significant difference in frailty status. This could also include strategies on optimal type of exercise and dose, when best to intervene for optimal outcomes and ways to reduce interruption to training taking into financial constraints face by the health service.

7.3 Telomere length outcomes in the study cohort

7.3.1 Age and gender effect on telomere length in dialysis cohort

Published data in healthy controls and non-CKD patients have consistently revealed that TL shortens with age. This inverse relationship was also observed in our study cohort with a statistically significant TL decline with age in our dialysis cohort. This observation was also seen in other published data of similar sample size in patients with ESKD. These findings reinforce the validity and reliability of RT-qPCR technique.

The present study has also shown that women who are on dialysis have a significantly longer TL in comparison to men who are on dialysis even after adjusting for age. However, this effect of gender was not seen in the control group with p=0.07. Despite the evidence that females have longer TL than males for a given chronological age, there have been 2 systematic reviews revealing that findings of TL in gender has not been consistent and not universally observed in studies that did not use Southern blot methods (Müezzinler, Zaineddin and Brenner, 2013) (Gardener and et al., 2014).

Longer TL in women has been attributed to the slower rate of TL attrition in women which is thought to be due to the high oestrogen exposure during the premenopausal period. There was an argument that similar findings many not been seen in women on dialysis due to alterations to their hormone panel leading to premature menopause. However, data from the Danish National Twin Registry has shown that regardless of

a woman's menopausal status, TL remains longer in women than men**.** Controversies on gender and TL remain; on the one hand, emerging evidence to suggests that female new-born babies may have longer telomeres than new-born males using flow fish (Aubert *et al.,* 2012) but not seen in another study that used TRF southern blotting (Okuda *et al.,* 2002).

7.3.2 Telomere length between dialysis patients and healthy controls

This study revealed that there was a difference between TL in the control group and ESKD group. However following adjustment for age and sex this significant association was lost. This was because the control group were younger than the dialysis group. This study failed to show any difference between TL; a marker of biological ageing to demonstrate that patients on dialysis age faster than healthy controls. The exact reason for this in unclear. The age group of the dialysis patients studied at our unit were younger than the average age of dialysis patients in the UK which is approximately 65 years. So perhaps this would account for the insignificant difference especially when measured with PCR technique between TL in healthy control and dialysis cohort. Studies which have revealed a difference between TL in healthy group and ESKD cohort had used FISH technique, Southern Blotting and G-tail telomeric length. The difference in TL measured with PCR technique is reported as a ratio and does not reflect the absolute length of telomeres and could underestimate the actual difference observed between the cohort (Refer to Chapter 8.2: Challenges and Limitations with Laboratory and Clinical Methodology on RT-qPCR technique for TL measurement and quality control measures). There is no consensus on

converting TL reported as T/S ratio to absolute kbp. The numbers have also been smaller than this study. Medications that dialysis patients are often on which include a statin, angiotensin converting enzyme and erythropoietin has been associated with longer TL (Tran, Meeker and Platz, 2018) (De Vries *et al.*, 2018)(Zhang, 2014). Therefore, it is possible that patients on dialysis had preservation of TL due to interventions in the form of medications and no difference was found between TL in healthy control and dialysis cohort.

This does not preclude current evidence suggesting that dialysis patients do have an accelerated ageing in comparison to healthy cohort but merely that TL may not be the ideal biomarker of ageing to demonstrate this difference or that the difference in TL due to uraemia is slow and as such this short term study may not capture this change.

7.3.3 Telomere length in haemodialysis and peritoneal dialysis

An area of interest in this study was to investigate if there were any differences between TL in HD and PD patients. The hypothesis was that a difference in TL may be observed in the different dialysis modality due to the different nature of the RRT process especially since TL is known to reflect oxidative stress. There has only been one study published to date that has attempted to compare TL in HD and PD cohorts. The study measured TL in $CD4^+$ and $CD8^+$ cells using flow fluorescence in situ hybridisation (FISH) technique. All the other studies published have only investigated

TL in HD patients only. There has been no published literature to date investigating TL in peritoneal dialysis patients with qPCR technique, therefore this work is unique in attempting to do so.

The results from this study failed to support the hypothesis that T/S ratio would be an ideal senescence marker representing premature senescence in ESKD cohort: as the mean T/S ratio between the HD and PD group showed no significant difference compared to the healthy controls. This outcome may also imply that T/S ratio; a form of a biomarker that reflects oxidative stress (Houben *et al.*, 2008) was similar in both groups. However, the study did not investigate and measure specific markers of oxidative stress that is associated with TL in uraemia (Tarng *et al.*, 2000)(Carrero *et al.*, 2008)(Murillo-Ortiz *et al.*, 2016). Meijers et.al; the only study comparing TL in HD and PD patients similarly found no statistically significant difference. These results may be due to HD and PD cohorts sharing many characteristics known to affect TL including age, gender, cardiovascular disease and uraemia. Data on 1-year mortality was similar between groups however the low number of events may mean that a small difference in mortality can't be excluded from this study due to a type 2 error. Besides, the increased survival data on PD was only seen in the early phases of starting PD and was influenced by multiple factors which include residual renal function which a large proportion of our patients had lost. However, the one interesting finding in this study is that the PD patients had T/S ratios on average of 0.0225 longer in comparison to HD patients following adjustment for age and sex at baseline suggesting there may be some influence of the modality itself affecting TL and may warrant further investigation in a larger prospective cohort study.

7.3.4 Dialysis vintage and telomere length

Prolonged dialysis exposure represented by dialysis vintage is a strong predictor of mortality as a result of multiple uraemic factors including increased oxidative stress and inflammation. Both oxidative stress and inflammation accelerate TL shortening leading to premature cellular senescence and reduced longevity as a result of accelerated biological ageing. These factors led us to believe that TL would be affected by dialysis vintage which was one of our hypotheses in this study.

However, our results have shown that there was no association between dialysis vintage and TL in our cohort irrespective of dialysis modality. There has been no data published previously on TL measured with qPCR and dialysis vintage in peritoneal dialysis cohort thus far. In fact, there has only been one study which had measured TL in 41 peritoneal dialysis patients using flow fluorescent in-site hybridisation (FISH) technique (Meijers *et al.*, 2012). Most published data investigating TL and dialysis vintage of HD patients have utilised other techniques mainly Southern blotting and FISH except one study that measured TL using qPCR which was published by Carrero et.al.

The results of dialysis vintage and TL from this study was comparable to results published by Carrero et.al that was similar in sample size and technique. Results from a study measuring TL in T-cells of ESKD patients showed that dialysis vintage was not significantly associated with TL (Meijers *et al.*, 2012). The duration of dialysis in this study cohort of 98 dialysis patients was between 0.1 and 22 years. The study

revealed that the younger dialysis patients with mean age of 35.7 ± 8.5 years had decreased TL in $CD8^+$ T-cells which may be accounted by the longer dialysis vintage with a mean of 4.3 years. However, these findings were not statistically significant. There have been several other published data supporting our initial hypothesis but the sample sizes in these studies were smaller and the techniques used to measure TL differed.

A study investigating the role of TL measured with Southern Blotting in 43 HD patients showed an inverse relationship between TL and dialysis vintage (Stefanidis *et al.*, 2015). The dialysis vintage in this study was 19.9 ± 15.6 months and the associations were only found between the 2 variables due to 4 outliers who have been on HD for 4-6 years. Another study using Southern Blotting from 38 HD patients revealed that TL was inversely proportional to time on dialysis but the mean/median dialysis vintage was not stated in the paper (Boxall *et al.*, 2006). The authors revealed that TL shortening was at 189bp per year of treatment on dialysis with the slope of the regression line for this relationship. Telomere shortening rate in healthy individuals are usually 20-40 bp/year (Lindsey *et al.*, 1991)(Vaziri *et al.*, 1994)(Boxall *et al.*, 2006).

Failure to confirm our hypothesis may be due to several factors; Probable measurement error in the assay when measuring TL in a small group of study cohort maybe a contributing factor as to the lack of consistent results among published data when addressing this hypothesis. With variable CV values reported in these studies which may seem small but do have a greater impact on the final outcome of T/S ratio.

The total sample size of dialysis patients who had TL measured successfully was 223. This study was a pilot study hence a power calculation was not done risking a type 2 error.

7.3.5 Telomere length and mortality

This study saw no association between TL and 1-year mortality as there was only 10 deaths within the 1 year of follow up risking a type 2 error. It may be interesting to evaluate TL and 5-year mortality data in this cohort as this is the median life expectancy for patients starting dialysis and as such, we would expect approaching 50% of patients who have remained on dialysis to have died in 5 years.

7.3.6 Telomere attrition in the case group

There are 3 longitudinal studies on TL changes in CKD and dialysis patients (Bansal *et al.*, 2012)(Luttropp *et al.*, 2016)(Kato *et al.*, 2016) . However, findings from these studies will need to be interpreted cautiously. The study by Bansal et.al found that kidney function was not independently associated with telomere shortening over 5 years, but the study participants had a mean eGFR (CKD-EPI) of 72.6 ± 21.5 ml/min/1.73m^2 and eGFR (cys) was 71.0 ± 23.2 ml/min/1.73m^2. The patients in this cohort were also relatively older with a mean age of 66.7 and stable coronary heart disease. A relevant study for comparison with this thesis findings reported that telomere attrition over 12 months in the dialysis group (n=49, HD=19 and PD=30) was small but still significant (Luttropp *et al.*, 2016). The decrease in TL was from

0.88 to 0.85 in this cohort. The authors also showed that a greater decrease in mean TL was observed after 12 months following renal transplantation. However, the study failed to show any association between age of the patients with TL at baseline.

The current study failed to show any significant difference in telomere attrition over a year using a mixed model approach. This is because the differences observed, even over several years, are very small and cannot truly be distinguished from measurement error or background noise. The general estimation for TL attrition is 30bp per year with the lengths of human telomeres estimated at 5-15kbp (so per year decline would be ~0.3%, current CVs (measurement error) are 4-5%).

Another major factor worth considering is the difference in the DNA sample storage times as the baseline DNA sample would have been stored for at least a year longer than DNA sample extracted at year 1. DNA storage conditions are known to impact quantitative PCR reaction. Dagnall et.al showed that samples that were stored at a higher concentration; 25ng/uL maintained strong correlation to the original results after 6 months but samples stored at 1ng/uL weekly correlated to the original samples (Dagnall *et al.*, 2017). There are no studies to show if these findings are largely unchanged in DNA stored longer than a year. There may be changes in DNA integrity that may not be detectable by using a spectrophotometer and the use of Qubit dsDNA Br Assay Kit or Quanti iT Picogreen dsDNA assay to measure double stranded DNA content of the sample may offer a solution (Lin *et al.*, 2019). The same DNA extraction facility and kit were used for DNA extraction from samples at baseline and 1 year as this is known to impact TL measured with qPCR (Lin *et al.*, 2019).

Measurement of TS ratio from an unpublished data of ~1000 individuals 10 years apart saw very little difference in TL for the majority of the cohort, which is what one would expect. This may explain the reason as to no significant change observed TL over 12 months in the study. The delta TS ratio of patients on PD was longer than HD patients but this likely be due to the fact the PD patients already had a baseline TL which was longer than HD.

7.3.7 Telomere length as a predictor of frailty in the case group

This is the first study to show a significant association between TL and frailty status in the dialysis patients. Increasing frailty was observed in dialysis patients with shorter TL which remained significant following multivariate logistic regression analysis. A ROC curve analysis showed that TL was a predictor of frailty with an AUC of 0.64.

There have been various studies in non-dialysis cohort demonstrating that TL is associated with frailty as reported in a recent systematic review of 10, 079 older adults (60 years and older) (Araújo Carvalho *et al.*, 2019). The review reported that frail older adults (n=355) had shorter telomeres than the non-frail (n = 1894) (Araújo Carvalho *et al.*, 2019).

The results of our work have demonstrated that TL may be a potential adjunct in identifying frail dialysis patients and may play a larger role in risk stratifying these patients with regards to mortality. In fact, results from this study support the use of TL analysis as an adjuvant biomarker which explains the intrinsic and multi system nature of the ageing process contributing to frailty.

A recent study demonstrated that a frailty measure constructed from standard laboratory tests and physiological parameters exhibits a significant association with TL among a cohort of the USA general population aged ≥60 years (Bello, Chiu and Dumancas, 2019). A combination of biomarkers of ageing and frailty reflecting this process across a range of physiological system may provide a better specificity and sensitivity than a single biomarker in predicting adverse health outcomes in a much more accurate manner especially in a complex group of patients e.g. dialysis patients.

The findings from this study is important in future research on frailty in uraemia as further investigations on the exact process and mechanism of TL shortening that eventually leads to frailty at a basic molecular level can be explored further.

7.4 Discussion of significant findings in ageing model of DNA methylation

7.4.1 DNA methylation and age

The findings of this study reveal that DNA methylation age had a strong correlation with age, r= 0.79 in the dialysis cohort (See Figure 40). Similar findings were shown by Hannum and Horvath with high age correlations (r=0.91 and r=0.96) and small, mean deviations from calendar age (4.9 and 3.6 years) (Hannum *et al.*, 2013)(Horvath, 2013). The mean deviation from calendar age in this study cohort was 11.3 years. Mean DNA methylation age was 1.78 years higher in the dialysis patients than their chronological age suggesting that dialysis patients have a higher biological age.

7.4.2 DNA methylation and frailty

The non- frail group had a mean DNA methylation age of 54.28 ± 14.6 in comparison to the frail group who had mean DNA methylation age of 61.41 ± 13.66. This difference was significant and supports our original hypothesis that frail dialysis patients' exhibit features consistent with accelerated ageing and that biological age may be a better predictor of frailty outcomes in dialysis patients than their chronological age. In this study we have used previously validated group of 71 CpG sites that have been shown to be highly predictive of age and built a model of methylation age based on a population of healthy controls. In applying this model to predict the ages of the study population, predicted methylation age referred to as DNA methylation age in this thesis as a surrogate marker of biological age.

Previous studies into methylation signatures of ageing have used AMAR or DNA methylation delta age as surrogate marker of biological age. However, in this study there was poor correlation with AMAR and DNA methylation delta age with chronological age as well as no association with frailty status. Both AMAR and DNA methylation delta age area derived from an individual's chronological age and may not be the best surrogate for chronological age. Therefore, to use either of the measures seems rather counter intuitive as the incorporation of chronological age might mask clinically significant associations that exist when the predicted methylation age is considered in isolation.

DNA methylation age was no longer significant with the development frailty following a multivariate analysis. As chronological age appeared to be a major contributor to this. Increasing DNA methylation age is significantly associated with

the development of frailty in the dialysis cohort though not superior to age based on the ROC curve analysis. Therefore, the current epigenetic signature for predicting methylation age is unlikely to be useful in terms of risk stratification as it was not possible to determine if these findings were causal or consequential or both. However, these findings provide a preliminary proof of concept validation that targeted methylation signatures are associated with frailty and may provide the basis of future research.

7.5 Comparison of results with previous studies

This is the first study looking into TL and DNAm as biomarkers of ageing to predict frailty as an outcome in the dialysis cohort. There are studies that have investigated TL in dialysis and DNAm in dialysis separately in other areas of nephrology but not specifically in frailty despite wide consensus on the major role frailty plays in dialysis. With regards to TL and ageing in dialysis, this would be the largest study in the dialysis patients and in particular peritoneal dialysis patients.

CHAPTER 8: CHALLENGES AND LIMITATIONS WITH LABORATORY AND CLINICAL METHODOLOGY

8.1 Fried frailty phenotype

There have been inconsistent outcomes in frailty measured in dialysis cohorts as a result of varying patient characteristics, different definitions of frailty and several ways of assessing frailty status. There is no consensus on the criteria, nor the scoring used when measuring frailty in uraemia. The chosen method to assess frailty for the purpose of this study was based on the Fried frailty phenotype which has been validated in the multi-ethnic dialysis population. Large scale population-based studies investigating frailty in dialysis patients were conducted in United States of America and often classify their patients into Caucasians, African American, Asian and others. The data available on the role and use of Fried frailty phenotype among Asians on dialysis is poorly described. Patient numbers in the Asian cohort are small in comparison to other ethnic groups which may account for lack of association or predictor of frailty.

One of the first studies investigating the association of frailty and ethnicity in non-dialysis/CKD patients showed that ethnicity was only related to frailty prevalence in

those aged 45 to 54 years with Caucasian participants being more likely to be frail than their African American counterparts (Griffin *et al.*, 2018). However this was not observed in other studies which reported an increase in frailty in African Americans aged between 65 and 79 years (Fried *et al.*, 2001) (Szanton *et al.*, 2010). A study from the UK investigating frailty by ethnic groups of patients >65 years of age based on demographic data revealed that the greatest prevalence of frailty was in the South Asian community (Pradhananga *et al.*, 2019). Prevalence of frailty was highest in the Bangladeshi population followed by Pakistanis and Indians (Pradhananga *et al.*, 2019).

Studies investigating frailty in the dialysis cohort showed black patients were less likely to be classified as frail in comparison to White Caucasians but the black participants were younger with mean age of 55 (Kutner, 2014). A study of 2275 dialysis patients showed that the 2.3% of Asian patients with mean ages of 58 were less likely to be frail in comparison to whites (Johansen *et al.*, 2007).

Black ethnicities have an increased proportion of muscle mass so one would expect a higher hand grip strength and reduced sarcopenia that would improve frailty. However, this was not the case in this study. There was no difference observed between hand grip strength and overall frailty scores between ethnic groups due to the small group numbers and unaccountable factors such as socioeconomic status.

As with all subjective assessments, the Fried frailty phenotype had several disadvantages which were encountered during this study, even though the questionnaire has been widely used in patients with ESKD previously. The

questionnaire depends on the patients being able to remember answers to the list of activities specified in the questionnaire. The activity questionnaire used in this study was based on the Minnesota Leisure Time Activity Questionnaire (MLTPAQ) which was utilised by Fried et.al. to calculate estimated energy expenditure. The questionnaire is elaborate and time consuming not to mention is subject to recall bias as patients are expected to report on frequencies and duration of each physical activity listed over a 12-month period. The MLTPAQ does not include more recent type of activities which patients engage with e.g. Zumba classes or fitness yoga. Many patients also keep track of their physical activity using their smart phones and apps mainly in the form of step counts. This meant that patients found it difficult to subsequently translate their step count to the required information on the physical activity questionnaire to enable energy expenditure calculation. Other studies have used various forms of questionnaires and assessments to assess the physical activity component. These include the use of LoPAQ (low physical activity questionnaire), PASE (physical activity scale for the Elderly), self-reported physical function using the physical function (PF) scale of the SF-36, self-reported questions on frequency of activity performed as well as the use of pedometers to measure physical activity in CKD cohort (Johansen *et al.*, 2015)(Johansen, 2015).

Patients also found it difficult to report on weight loss, as often the reported weight loss was related to fluid removal rather than actual flesh weight. The Fried frailty phenotype methodology used for the purpose of this study measured weakness objectively through grip strength which requires the body mass index to assess the cut off for grip strength when determining ones' frailty score. Often an overhydrated volume status leads to overestimation of body weight which results with a higher BMI

affecting end scores for frailty domains. A similar issue was encountered when assessing weight of PD patients as due to the nature of their dialysis, these patients often have dialysis fluid in their peritoneum at all times. This was addressed by establishing their weight at clinic when there is no fluid in the peritoneum or by assessing their dry weight based on the known amount of fluid infused into the peritoneum.

8.2 RT-qPCR technique for TL measurement and quality control measures

The technique utilised for the purpose of this PhD has many advantages and is the preferred choice for large population-based studies due to its high throughput. However, despite the fact that this technique is well established, there are some limitations which could potentially affect the outcome of this study which this PhD has tried to address to its best.

One of the main challenges has been the reproducibility of the technique which is affected by several factors including pipetting errors and DNA quality. The RT-qPCR assay to measure TL needs to have high quality DNA that is not compromised by degradation. This is addressed by evaluating each sample by gel electrophoresis. The assay precision is also affected by small changes in efficiency in earlier cycles which may result in larger changes due to amplification in subsequent cycles. These changes in efficiency is caused by primers mis-priming, changes in thermal cycling temperature and reagents, which are minimised by using a control DNA acting as a

positive control. A negative control is included on each run to assess for any unexpected amplification or contamination in the assay.

Cawthon, who first described this technique to measure TL had observed an inter-assay coefficient variation of 5.8% and an intra-assay coefficient variation of 13.8% for triplicates and 3.4% for duplicates. The inter-assay CV in this study was 4.86% whilst the intra-assay CV for the Tel run was 1.57% and 1.51% for SCG run. Samples in this study were performed in duplicates. The intra-assay CV of both Tel and SCG run in this study was lower than the intra-assay CV observed by Cawthon as this study excluded any samples where the duplicate values were more than 0.2 of a cycle differences for the take-off value.

Other disadvantages of RT-qPCR are that the results do not provide an absolute kilo base length estimate of TL, but a relative TL or better known as T/S ratio. This proxy measurement however has been shown to be an accurate representation of absolute TL. Another disadvantage of this technique is the difficulty in directly comparing different samples between studies due to probable differences in the DNA quality and DNA extraction methods unless this information is known.

8.3 DNA methylation and limitations

There are several strength and limitations to the current study. A significant strength of the study is that DNA methylation status is gaining wider interest and has been postulated as a better biological marker. In fact, the role of individual CpG sites from DNA methylation analysis may be able to offer more information in clinical studies which can set the platform for investigating the effect of intervention based on the changes of these CpG sites. There are currently 850,000 CpG sites mapped in the EPIC methylation array and 450,000 sites mapped in the Illumina Human Methylation 450 bead chip. This study mapped 71 CpG sites instead of the mapping the CpG sites described as a targeted approach which has been well established. There is increasing literature on targeted identification of CpG sites to help reduce cost when using an epigenetic marker for a study. Firstly, not all of the 71 of the ageing signature CpG sites of interest or the 6 CpG sites described by Koch et.al as correlating with cellular senescence was successfully mapped in this study. This was due to a failure to design primers specific enough to amplify the CpG of interest. This is one of the limitations of CpG target specific approach. Due to this only 48 of the 71 CpG sites were used to generate the model used to calculate the methylation age in this study cohort. However, despite this limitation the model correlated well with the chronological age of healthy donors with an RMSE of 6.89, R= 0.75. The loss of the original CpG sites described by Hannum was not a limitation for this study but it is important to note that the final model used in the analysis for this study was based on the Hannum epigenetic clock but not the exact same signature as described in his paper (Hannum *et al.*, 2013). Another potential limitation is that DNA methylation analysis requires the creation of

a mathematical model to estimate DNA methylation age in the group of interest. The model is built based on the CpG sites from healthy donors. Therefore, it is not surprising that DNA methylation has a strong correlation with chronological age. There is increasing interest in investigating specific target CpG sites for ageing which may provide a targeted approach in investigating ageing in a cohort of interest as the CpG sites may differ based on underlying clinical condition.

CHAPTER 9: CONCLUSION AND FUTURE WORK

9.1 Summary of key findings

From the results of this study the following conclusions can be drawn:

1) Frailty was higher among HD patients in comparison to PD patients and this difference was significant, p= 0.015. The relative risk of frailty was 1.69 times higher in HD than in PD and this was significant, p=0.0122.

2) A univariate analysis revealed that mean TL was shorter in ESKD patients in comparison to healthy controls, but the significance was lost following a multivariate analysis adjusting for age and sex as potential confounders.

3) Telomere attrition was borderline significant, p=0.05 with patients in the PD group having a mean estimate of ΔT/S by -0.060 i.e. less telomere attrition or accelerated ageing.

4) No significant association between mortality outcomes and TL was observed, though the direction of effect is consistent with expectations, i.e. longer TL is associated with less risk of death

5) TL is associated with frailty status. The risk of frailty on dialysis is increased by 52.2% for each reduction of telomere length by one SD. TL remained to be an independent predictor of frailty status when adjusted for 12 variables.

6) DNA methylation age was significantly higher in the frail group in comparison to the non-frail group with a univariate analysis, but this association was lost in a multivariate analysis.

9.2 Clinical impact of these findings

These results from this single-centre prospective pilot study suggest that TL; a novel biomarker of ageing and senescence may be able to better predict frailty status in ESKD cohort. The study also showed that telomere attrition difference in both the dialysis modalities reached near significance with p=0.05 with reduced degree of telomere attrition in the PD group. Both these findings are crucial and relevant in ageing among dialysis population. More importantly these outcomes will pave the way for a larger study to be conducted in this area/subject matter. More longitudinal studies are required to investigate the role of TL and telomere attrition as the current study was not be able to comment if the findings on TL and frailty were causal or consequential.

It is interesting that despite TL was correlated with methylome age, only TL had revealed significant association with frailty status which no doubt is worth exploring

into. There may be a different pathophysiology on the way these genetic and epigenetic ageing biomarkers function.

Findings as revealed in this study can obviously have potential clinical impact as many decisions in the field of nephrology is determined by chronological age e.g. consideration for dialysis initiation, advanced care planning, listing for renal transplantation and escalation of care. The self-reported age of a dialysis patient may not be a true reflection of their chronological age as a large proportion of these patients were not born in the UK; with the possibility of having an incorrect birth date due to missing identification during settlement process (Anderson, Herr and McCowan, 2013). Incorrect chronological age will certainly have an impact on adjusted data analysis and correlation analysis for TL, DNAm and frailty scores. Both TL and DNAm parameters are correlated with age and all data presented in this study were adjusted for chronological age as a confounder. Therefore, the extent of incorrect chronological age and the impact on analysis will be difficult to ascertain. Performing all analysis by excluding patients who were born outside UK will not be possible due to the small numbers.

Often many of the well-established risk scoring systems used in the general population are not validated in dialysis patients. This also includes the limited options for measuring frailty in the dialysis patients and the accuracy of these measurements having observed the limitations and difficulties faced by my study recruits when completing the assessments. It is possible that a personalised measure of genetic or epigenetic age might offer a better predictive power in models taking into account other clinical parameter to measure frailty risk in the dialysis patients.

9.3 Potential future analysis

Results from this study raise the possibility of investigating the role of TL and DNAm further not only to the limit of predicting frailty outcomes but also the individual components of interest. Ageing and frailty are important factors in the field of dialysis and transplantation and investigating the role of genetic and epigenetic ageing markers in this field is clinically relevant.

There may be relationships and associations between the variables collected during the study which may become more apparent and significant with further follow up of this cohort. Whilst no statistical significance was seen in TL at baseline and death, but the direction of effect was consistent with an increased risk of death with shorter TL in the dialysis cohort. A longer follow up duration might also result in us detecting a difference in morbidity and mortality outcomes based on frailty status, TL, telomere attrition rate and DNA methylation-based parameters. As such, significant difference might become apparent on longer follow up with increased number of endpoints. The current cohort will be followed up to 5 and 10 years for mortality.

DNA based methylation parameters were inferior to TL in predicting frailty status in the current study population. However, there is increased interest in the role targeted and individual specific CpG sites instead of using DNA methylome parameters to correlate with clinical outcomes. as the status at specific sites may be more accurate. Data from 6 CpG sites correlating with cellular senescence is available and there is an idea to analyse these methylation beta values against frailty and mortality outcomes in future which may lead to interesting findings.

Presentations to Learned Societies

1. Does Peritoneal Dialysis Vintage Affect Relative Leucocyte Telomere Length: A Novel Biomarker of Ageing? Vasantha M Muthuppalaniappan, Damian C Balmforth, Julius E Kieswich, Kieran McCafferty, Muhammad Magdi Yaqoob.

   ERA-EDTA. Copenhagen 2018 and UK Kidney Week Brighton 2019

2. Is Pre-renal Transplant Frailty and Telomere Length Associated with Length of Stay and Hospital Re-admissions Post Transplantations? Vasantha M Muthuppalaniappan, Damian C Balmforth, Julius E Kieswich, Kieran McCafferty, Muhammad Magdi Yaqoob. ERA-EDTA. Copenhagen 2018 and UK Kidney Week 2019

Publications

1. Muthuppalaniappan VM, Balmforth D, McCafferty K, Yaqoob MM. Is Pre-renal Transplant Frailty and Telomere Length Associated with Length of Stay and Hospital Readmissions Post Transplantation? [Abstract FP736]. *Nephrology Dialysis Transplantation*. 2018 (May); Volume 33: Issue suppl_1, Page i294
   https://doi.org/10.1093/ndt/gfy104.FP736

2. Muthuppalaniappan VM, Kieswich J, McCafferty K, Yaqoob MM. Does Peritoneal Dialysis Vintage Affect Relative Leucocyte Telomere Length; A Novel Biomarker of Ageing? [Abstract FP473]. *Nephrology Dialysis Transplantation*, 2018 (May); Volume 33: Issue suppl_1, Page i196,
   https://doi.org/10.1093/ndt/gfy104.FP473

References

Abellan Van Kan, G. *et al.* (2009) 'Gait speed at usual pace as a predictor of adverse outcomes in community-dwelling older people an International Academy on Nutrition and Aging (IANA) task force', *Journal of Nutrition, Health and Aging*. doi: 10.1007/s12603-009-0246-z.

Aeby, E. *et al.* (2016) 'Peroxiredoxin 1 Protects Telomeres from Oxidative Damage and Preserves Telomeric DNA for Extension by Telomerase Report Peroxiredoxin 1 Protects Telomeres from Oxidative Damage and Preserves Telomeric DNA for Extension by Telomerase', *CellReports*. ElsevierCompany., 17(12), pp. 3107–3114. doi: 10.1016/j.celrep.2016.11.071.

Ahmed, W. and Lingner, J. (2018) 'Impact of oxidative stress on telomere biology', *Differentiation*. Elsevier B.V., 99(October 2017), pp. 21–27. doi: 10.1016/j.diff.2017.12.002.

Alem, A. M. *et al.* (2000) 'Increased risk of hip fracture among patients with end-stage renal disease', *Kidney International*. doi: 10.1046/j.1523-1755.2000.00178.x.

Anderson, G., Herr, K. and McCowan, T. (2013) *Child Marriage in Bangladesh: Birth and Marriage Registration*, *Encyclopedia of Activism and Social Justice*. doi: 10.4135/9781412956215.n885.

Araújo Carvalho, A. C. *et al.* (2019) 'Telomere length and frailty in older adults—A systematic review and meta-analysis', *Ageing Research Reviews*, 54(February). doi: 10.1016/j.arr.2019.100914.

Armanios, M. and Blackburn, E. H. (2012) 'The telomere syndromes', *Nature Reviews Genetics*. Nature Publishing Group, a division of Macmillan Publishers Limited. All

Rights Reserved., 13, p. 693. Available at: http://dx.doi.org/10.1038/nrg3246.

Arneson, T. J. *et al.* (2013) 'Trends in hip fracture rates in US hemodialysis patients, 1993-2010', *American Journal of Kidney Diseases*. doi: 10.1053/j.ajkd.2013.02.368.

Aubert, G. *et al.* (2012) 'Collapse of Telomere homeostasis in hematopoietic cells caused by heterozygous mutations in Telomerase genes', *PLoS Genetics*. doi: 10.1371/journal.pgen.1002696.

Aviv, A. (2002) 'Telomeres, sex, reactive oxygen species, and human cardiovascular aging', *Journal of Molecular Medicine*. doi: 10.1007/s00109-002-0377-8.

Aviv, A. *et al.* (2005) 'The longevity gender gap: are telomeres the explanation?', *Science of aging knowledge environment : SAGE KE*. doi: 10.1126/sageke.2005.23.pe16.

Bansal, N. *et al.* (2012) 'Association between kidney function and telomere length: The heart and soul study', *American Journal of Nephrology*, 36(5), pp. 405–411. doi: 10.1159/000343495.

Bao, Y. *et al.* (2012) 'Frailty, dialysis initiation, and mortality in end-stage renal disease', *Archives of Internal Medicine*, 172(14), pp. 1071–1077. doi: 10.1001/archinternmed.2012.3020.

Barrett, E. L. B. and Richardson, D. S. (2011) 'Sex differences in telomeres and lifespan', *Aging Cell*. doi: 10.1111/j.1474-9726.2011.00741.x.

Bataille, S. *et al.* (2017) 'The diagnosis of sarcopenia is mainly driven by muscle mass in hemodialysis patients', *Clinical Nutrition*. doi: 10.1016/j.clnu.2016.10.016.

Baylis, D. *et al.* (2014) 'Inflammation, telomere length, and grip strength: A 10-year longitudinal study', *Calcified Tissue International*, 95(1), pp. 54–63. doi: 10.1007/s00223-014-9862-7.

Bayne, S. and Liu, J. P. (2005) 'Hormones and growth factors regulate telomerase

activity in ageing and cancer', *Molecular and Cellular Endocrinology*, 240(1–2), pp. 11–22. doi: 10.1016/j.mce.2005.05.009.

Bekaert, S. *et al.* (2007) 'Telomere length and cardiovascular risk factors in a middle-aged population free of overt cardiovascular disease', *Aging Cell*. doi: 10.1111/j.1474-9726.2007.00321.x.

Bellizzi, D. *et al.* (2012) 'Global DNA methylation levels are modulated by mitochondrial DNA variants', *Epigenomics*. doi: 10.2217/epi.11.109.

Bello, G. A., Chiu, Y. M. and Dumancas, G. G. (2019) 'Association of a biomarker-based frailty index with telomere length in older US adults : Findings from the National Health and Nutrition Examination Survey 1999–2002', *EMJ Innov*, 3(1), pp. 73–81. doi: 10.1101/191023.

Belsky, D. W. *et al.* (2015) 'Quantification of biological aging in young adults', *Proceedings of the National Academy of Sciences*, 112(30), pp. E4104–E4110. doi: 10.1073/pnas.1506264112.

Bendix, L. *et al.* (2014) 'Longitudinal changes in leukocyte telomere length and mortality in humans', *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*. doi: 10.1093/gerona/glt153.

Benetos, a *et al.* (2001) 'Telomere length as an indicator of biological aging: the gender effect and relation with pulse pressure and pulse wave velocity.', *Hypertension*, 37(2 Part 2), pp. 381–385. doi: 10.1161/01.HYP.37.2.381.

Benetos, A. *et al.* (2018) 'Short leukocyte telomere length precedes clinical expression of atherosclerosis the blood-And-muscle model', *Circulation Research*. doi: 10.1161/CIRCRESAHA.117.311751.

Bernadotte, A., Mikhelson, V. M. and Spivak, I. M. (2016) 'Markers of cellular senescence. Telomere shortening as a marker of cellular senescence', *Aging*, 8(1), pp.

3–11. doi: 10.18632/aging.100871.

Betjes, M. G. H. *et al.* (2011) 'Premature aging of circulating T cells in patients with end-stage renal disease', *Kidney International*. Elsevier Masson SAS, 80(2), pp. 208–217. doi: 10.1038/ki.2011.110.

Bird, A. (2002) 'DNA methylation patterns and epigenetic memory', *Genes and Development*. doi: 10.1101/gad.947102.

Blackburn, E. H., Greider, C. W. and Szostak, J. W. (2009) 'Advanced information: Maintenance of chromosomes by telomeres and the enzyme telomerase', *Nobel Prize in Physiology or Medicine 2009*, 1(12), pp. 1–12. Available at: http://www.nobelprize.org/nobel_prizes/medicine/laureates/2009/advanced.html.

Blasco, M. *et al.* (1997) 'Telomere shortening and tumor formation by mouse cells lacking telomerase RNA.', *Cell*, 91(1), pp. 25–34. doi: S0092-8674(01)80006-4 [pii].

Boonekamp, J. J. *et al.* (2014) 'Nestling telomere shortening, but not telomere length, reflects developmental stress and predicts survival in wild birds', *Proceedings of the Royal Society B: Biological Sciences*. doi: 10.1098/rspb.2013.3287.

Boonekamp, J. J. *et al.* (2017) 'Does oxidative stress shorten telomeres?', *Biology Letters*. doi: 10.1098/rsbl.2017.0164.

Borras, M. *et al.* (2012) 'Assessment of the Potential Role of Active Vitamin D Treatment in Telomere Length: A Case-Control Study in Hemodialysis Patients', *Clinical Therapeutics*. doi: 10.1016/j.clinthera.2012.02.016.

Bossola, M. *et al.* (2015) 'Fatigue is associated with serum interleukin-6 levels and symptoms of depression in patients on chronic hemodialysis', *Journal of Pain and Symptom Management*. doi: 10.1016/j.jpainsymman.2014.07.009.

Boxall, M. C. *et al.* (2006) 'Telomere shortening and haemodialysis', *Blood Purification*, 24(2), pp. 185–189. doi: 10.1159/000090517.

Brault, M. E. *et al.* (2014) 'Telomere Length and the Clinical Phenotype of Frailty in Older Adults Undergoing Cardiac Surgery', *Journal of the American Geriatrics Society*. doi: 10.1111/jgs.13076.

Breitling, L. P. *et al.* (2016) 'Frailty is associated with the epigenetic clock but not with telomere length in a German cohort', *Clinical Epigenetics*. Clinical Epigenetics, 8(1), pp. 1–8. doi: 10.1186/s13148-016-0186-5.

Bressler, J. *et al.* (2016) 'DNA methylation - based measures of biological age : meta - analysis predicting time to death', *Aging*. doi: 10.18632/aging.101020.

Brouilette, S. W. *et al.* (2007) 'Telomere length, risk of coronary heart disease, and statin treatment in the West of Scotland Primary Prevention Study: a nested case-control study', *Lancet*, 369(9556), pp. 107–114. doi: 10.1016/S0140-6736(07)60071-3.

Buta, B. J. *et al.* (2016) 'Frailty assessment instruments: Systematic characterization of the uses and contexts of highly-cited instruments', *Ageing Research Reviews*, pp. 53–61. doi: 10.1016/j.arr.2015.12.003.

Buxton, J. L. *et al.* (2014) 'Human leukocyte telomere length is associated with DNA methylation levels in multiple subtelomeric and imprinted loci', *Scientific Reports*, 4, pp. 1–8. doi: 10.1038/srep04954.

Canela, A. *et al.* (2007) 'High-throughput telomere length quantification by FISH and its application to human population studies', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.0609367104.

Carrero, J. J. *et al.* (2008) 'Telomere attrition is associated with inflammation, low fetuin-A levels and high mortality in prevalent haemodialysis patients', *Journal of Internal Medicine*, 263(3), pp. 302–312. doi: 10.1111/j.1365-2796.2007.01890.x.

Carrero, J. J. (2009) 'Identification of Patients With Eating Disorders: Clinical and

Biochemical Signs of Appetite Loss in Dialysis Patients', *Journal of Renal Nutrition*. doi: 10.1053/j.jrn.2008.10.004.

Cawthon, R. *et al.* (2003) 'Association between telomere length in blood and mortality in people aged 60 years or older', *The Lancet*, 361(9355), pp. 393–395. doi: 10.1016/S0140-6736(03)12384-7.

Cawthon, R. M. (2002) 'Telomere measurement by quantitative PCR.', *Nucleic acids research*, 30(10), p. e47. doi: 10.1093/nar/30.10.e47.

Cawthon, R. M. (2009) 'Telomere length measurement by a novel monochrome multiplex quantitative PCR method', *Nucleic Acids Research*, 37(3), pp. 1–7. doi: 10.1093/nar/gkn1027.

Cesari, M. *et al.* (2004) 'Inflammatory markers and physical performance in older persons: the InChianti study', *J.Gerontol.A Biol.Sci.Med Sci.*, 59(3), pp. 242–248.

Chaves, P. H. *et al.* (2005) 'Impact of anemia and cardiovascular disease on frailty status of community-dwelling older women: the Women's Health and Aging Studies I and II', *The journals of gerontology.Series A, Biological sciences and medical sciences*, 60(6), pp. 729–735.

Chen, B. H. *et al.* (2016) 'DNA methylation-based measures of biological age: Meta-analysis predicting time to death', *Aging*. doi: 10.18632/aging.101020.

Chen, B. H. *et al.* (2017) 'Leukocyte telomere length, T cell composition and DNA methylation age', *Aging*. doi: 10.18632/aging.101293.

Chen, Q. I. N. *et al.* (1995) 'Oxidative DNA damage and senescence of human diploid fibroblast cells.', *PNAS*, 92(May), pp. 4337–4341.

Chen, W. *et al.* (2011) 'Longitudinal versus cross-sectional evaluations of leukocyte telomere length dynamics: Age-dependent telomere shortening is the rule', *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, 66 A(3), pp.

312–319. doi: 10.1093/gerona/glq223.

Cherkas, L. *et al.* (2008) 'The association between physical activity in leisure time and leukocyte telomere length.', *Archives of internal medicine*, 168(2), pp. 154–158. doi: 10.1001/archinternmed.2007.39.

Clark, D. A. *et al.* (2017) 'Frailty in end-stage renal disease: comparing patient, caregiver, and clinician perspectives', *BMC Nephrology*. BMC Nephrology, 18(1), pp. 1–8. doi: 10.1186/s12882-017-0558-x.

Clegg, A. *et al.* (2013) 'Frailty in elderly people', in *The Lancet*, pp. 752–762. doi: 10.1016/S0140-6736(12)62167-9.

Cohen, H. J., Harris, T. and Pieper, C. F. (2003) 'Coagulation and activation of inflammatory pathways in the development of functional decline and mortality in the elderly', *American Journal of Medicine*, 114(3), pp. 180–187. doi: 10.1016/S0002-9343(02)01484-5.

Collard, R. M. *et al.* (2012) 'Prevalence of frailty in community-dwelling older persons: A systematic review', *Journal of the American Geriatrics Society*. doi: 10.1111/j.1532-5415.2012.04054.x.

Collerton, J. *et al.* (2012) 'Frailty and the role of inflammation , immunosenescence and cellular ageing in the very old : Cross-sectional findings from the Newcastle 85 + Study', *Mechanisms of Ageing and Development*. Elsevier Ireland Ltd, 133(6), pp. 456–466. doi: 10.1016/j.mad.2012.05.005.

Collerton, J. *et al.* (2014) 'Acquisition of aberrant DNA methylation is associated with frailty in the very old: Findings from the Newcastle 85+ Study', *Biogerontology*. doi: 10.1007/s10522-014-9500-9.

Crepin, T. *et al.* (2015) 'ATG-induced accelerated immune senescence: Clinical implications in renal transplant recipients', *American Journal of Transplantation*. doi:

10.1111/ajt.13092.

Crépin, T. *et al.* (2018) 'Uraemia-induced immune senescence and clinical outcomes in chronic kidney disease patients', *Nephrology Dialysis Transplantation*. doi: 10.1093/ndt/gfy276.

Currais, A. (2017) 'The origin of life at the origin of ageing?', *Ageing Research Reviews*. Elsevier B.V., 35, pp. 297–300. doi: 10.1016/j.arr.2016.10.007.

Dagnall, C. L. *et al.* (2017) 'Effect of pre-analytic variables on the reproducibility of qPCR relative telomere length measurement', *PLoS ONE*. doi: 10.1371/journal.pone.0184098.

Daniali, L. *et al.* (2013) 'Telomeres shorten at equivalent rates in somatic tissues of adults', *Nature Communications*. doi: 10.1038/ncomms2602.

Darío, M.-E. J. *et al.* (2019) 'Oxidative stress, telomere length, and frailty in an old age population', *bioRxiv*, p. 414680. doi: 10.1101/414680.

Demissie, S. *et al.* (2006) 'Insulin resistance, oxidative stress, hypertension, and leukocyte telomere length in men from the Framingham Heart Study', *Aging Cell*, 5(4), pp. 325–330. doi: 10.1111/j.1474-9726.2006.00224.x.

DiMatteo, M. R., Lepper, H. S. and Croghan, T. W. (2000) 'Depression is a risk factor for noncompliance with medical treatment meta-analysis of the effects of anxiety and depression on patient adherence', *Archives of Internal Medicine*. doi: 10.1001/archinte.160.14.2101.

Donovan, A. O. *et al.* (2011) 'Cumulative Inflammatory Load Is Associated with Short Leukocyte Telomere Length in the Health , Aging and Body Composition Study', 6(5). doi: 10.1371/journal.pone.0019687.

Epel, E. S. *et al.* (2004) 'Accelerated telomere shortening in response to life stress', *Proceedings of the National Academy of Sciences*, 101(49), pp. 17312–17315. doi:

10.1073/pnas.0407162101.

Epel, E. S. *et al.* (2009) 'The rate of leukocyte telomere shortening predicts mortality from cardiovascular disease in elderly men.', *Aging*.

Feinberg, A. P. (2007) 'Phenotypic plasticity and the epigenetics of human disease', *Nature*. doi: 10.1038/nature05919.

Feinberg, A. P., Koldobskiy, M. A. and Göndör, A. (2016) 'Epigenetic modulators, modifiers and mediators in cancer aetiology and progression', *Nature Reviews Genetics*. doi: 10.1038/nrg.2016.13.

Felsenfeld, G. (2014) 'A brief history of epigenetics', *Cold Spring Harbor Perspectives in Biology*. doi: 10.1101/cshperspect.a018200.

Ferrucci, L. and Fabbri, E. (2018) 'Inflammageing: chronic inflammation in ageing, cardiovascular disease, and frailty', *Nature Reviews Cardiology*, 15(9), pp. 505–522. doi: 10.1038/s41569-018-0064-2.

Fraga, M. *et al.* (2004) 'From The Cover: Epigenetic differences arise during the lifetime of monozygotic twins', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.0500398102.

Fransquet, P. D. *et al.* (2019) 'The epigenetic clock as a predictor of disease and mortality risk: A systematic review and meta-analysis', *Clinical Epigenetics*. Clinical Epigenetics, 11(1), pp. 1–17. doi: 10.1186/s13148-019-0656-7.

Franzen, J. *et al.* (2017) 'Senescence-associated DNA methylation is stochastically acquired in subpopulations of mesenchymal stem cells', *Aging Cell*. doi: 10.1111/acel.12544.

Frenck, R. W., Blackburn, E. H. and Shannon, K. M. (1998) 'The rate of telomere sequence loss in human leukocytes varies with age', *Proceedings of the National Academy of Sciences of the United States of America*, 95(10), pp. 5607–5610. doi:

10.1073/pnas.95.10.5607.

Fried, L. P. *et al.* (2001) 'Frailty in Older Adults: Evidence for a Phenotype', *Journal of Gerontology: MEDICAL SCIENCES America*, 56(3), pp. 146–156. doi: 10.1093/gerona/56.3.M146.

Gale, C. R. *et al.* (2018) 'DNA methylation and the epigenetic clock in relation to physical frailty in older people: The Lothian Birth Cohort 1936', *Clinical Epigenetics*. Clinical Epigenetics, 10(1), pp. 1–8. doi: 10.1186/s13148-018-0538-4.

Gallagher, D. *et al.* (2000) 'Weight stability masks sarcopenia in elderly men and women.', *American journal of physiology. Endocrinology and metabolism*. doi: 10.1093/gerona/50a.special_issue.1.

Gama-sosa, M. A. *et al.* (1983) 'The 5-methylcytosine content of DNA from human tumors', *Nucleic Acids Research*. doi: 10.1093/nar/11.19.6883.

Gardano, L. *et al.* (2012) 'Native gel electrophoresis of human telomerase distinguishes active complexes with or without dyskerin', *Nucleic Acids Research*, 40(5). doi: 10.1093/nar/gkr1243.

Gardener, M. and et al. (2014) 'Gender and telomere length: Systematic review and meta- analysis ☆ HHS Public Access', *Exp Gerontol*, 51, pp. 15–27. doi: 10.1016/j.exger.2013.12.004.

Gensous, N. *et al.* (2017) 'The epigenetic landscape of age-related diseases: the geroscience perspective', *Biogerontology*. doi: 10.1007/s10522-017-9695-7.

Ghattas, M. *et al.* (2014) 'DNA methylation status of the methylenetetrahydrofolate reductase gene promoter in peripheral blood of end-stage renal disease patients', *Molecular Biology Reports*, 41(2), pp. 683–688. doi: 10.1007/s11033-013-2906-7.

Goglin, S. E. *et al.* (2016) 'Change in leukocyte telomere length predicts mortality in

patients with stable coronary heart disease from the heart and soul study', *PLoS ONE*. doi: 10.1371/journal.pone.0160748.

Gonzalo, S. *et al.* (2006) 'DNA methyltransferases control telomere length and telomere recombination in mammalian cells.', *Nature cell biology*. doi: 10.1038/ncb1386.

Griffin, F. R. *et al.* (2018) 'Frailty in a racially and socioeconomically diverse sample of middle-aged Americans in Baltimore', *PLoS ONE*. doi: 10.1371/journal.pone.0195637.

Griffith, J. D. *et al.* (1999) 'Mammalian telomeres end in a large duplex loop', *Cell*, 97(4), pp. 503–514. doi: 10.1016/S0092-8674(00)80760-6.

Haapanen, M. J. *et al.* (2018) 'Telomere Length and Frailty: The Helsinki Birth Cohort Study', *Journal of the American Medical Directors Association*. doi: 10.1016/j.jamda.2018.05.011.

Hanlon, P. *et al.* (2018) 'Frailty and pre-frailty in middle-aged and older adults and its association with multimorbidity and mortality: a prospective analysis of 493 737 UK Biobank participants', *The Lancet Public Health*. The Author(s). Published by Elsevier Ltd. This is an Open Access article under the CC BY 4.0 license, 3(7), pp. e323–e332. doi: 10.1016/S2468-2667(18)30091-4.

Hannum, G. *et al.* (2013) 'Genome-wide Methylation Profiles Reveal Quantitative Views of Human Aging Rates', *Molecular Cell*. doi: 10.1016/j.molcel.2012.10.016.

Harley, C. B. (1991) 'Telomere loss: mitotic clock or genetic time bomb?', *Mutation Research DNAging*, 256(2–6), pp. 271–282. doi: 10.1016/0921-8734(91)90018-7.

Harley, C. B., Futcher, B. A. and Greider, C. W. (1990) 'Telomeres Shorten during aging fibroblasts_Harley C_Nature_1990.pdf', *Nature*. Available at: https://www.nature.com/articles/345458a0.pdf.

van der Harst, P. *et al.* (2008) 'Possible Association Between Telomere Length and Renal Dysfunction in Patients With Chronic Heart Failure', *American Journal of Cardiology*, 102(2), pp. 207–210. doi: 10.1016/j.amjcard.2008.03.040.

Hayflick, L. (1965) 'The limited in vitro lifetime of human diploid cell strains', *Experimental Cell Research*, 37(3), pp. 614–636. doi: 10.1016/0014-4827(65)90211-9.

Hayflick, L. (1979) 'The cell biology of aging', *Journal of Investigative Dermatology*. Elsevier Masson SAS, 73(1), pp. 8–14. doi: 10.1111/1523-1747.ep12532752.

Hayflick, L. and Moorhead, P. (1961) 'THE SERIAL CULTIVATION OF HUMAN DIPLOID CELL STRAINS', 1.

Herbstman, J. B. *et al.* (2013) 'Predictors and Consequences of Global DNA Methylation in Cord Blood and at Three Years', *PLoS ONE*. doi: 10.1371/journal.pone.0072824.

Himmelfarb, J. *et al.* (2002) 'Perspectives in renal medicine: The elephant in uremia: Oxidant stress as a unifying concept of cardiovascular disease in uremia', *Kidney International*, 62(5), pp. 1524–1538. doi: 10.1046/j.1523-1755.2002.00600.x.

Hirashio, S. *et al.* (2014) 'Telomeric G-Tail length and hospitalization for cardiovascular events in hemodialysis patients', *Clinical Journal of the American Society of Nephrology*, 9(12), pp. 2117–2122. doi: 10.2215/CJN.10010913.

Honda, H. *et al.* (2007) 'Obese sarcopenia in patients with end-stage renal disease is associated with inflammation and increased mortality', *American Journal of Clinical Nutrition*, 86(3), pp. 633–638.

Horvath, S. (2013) 'DNA methylation age of human tissues and cell types.', *Genome biology*, 14(10), p. R115. doi: 10.1186/gb-2013-14-10-r115.

Horvath, S. *et al.* (2014) 'Obesity accelerates epigenetic aging of human liver',

Proceedings of the National Academy of Sciences. doi: 10.1073/pnas.1412759111.

Houben, J. M. J. *et al.* (2008) 'Telomere length assessment: Biomarker of chronic oxidative stress?', *Free Radical Biology and Medicine*, 44(3), pp. 235–246. doi: 10.1016/j.freeradbiomed.2007.10.001.

Hsu, C. Y. *et al.* (2012) 'Global DNA methylation not increased in chronic hemodialysis patients: A case-Control study', *Renal Failure*. doi: 10.3109/0886022X.2012.723280.

Hubbard, R. E. *et al.* (2009) 'Inflammation and frailty measures in older people', *Journal of Cellular and Molecular Medicine*, 13(9 B), pp. 3103–3109. doi: 10.1111/j.1582-4934.2009.00733.x.

Jassal, S. V., Chiu, E. and Hladunewich, M. (2009) 'Loss of independence in patients starting dialysis at 80 years of age or older.', *The New England journal of medicine*, 361(16), pp. 1612–1613. doi: 10.1056/NEJMc0905289.

Jennings, P. *et al.* (2007) 'Cyclosporine A induces senescence in renal tubular epithelial cells', *American Journal of Physiology - Renal Physiology*, 293(3), pp. 831–838. doi: 10.1152/ajprenal.00005.2007.

Jhamb, M. *et al.* (2008) 'Fatigue in Patients Receiving Maintenance Dialysis: A Review of Definitions, Measures, and Contributing Factors', *American Journal of Kidney Diseases*, 52(2), pp. 353–365. doi: 10.1053/j.ajkd.2008.05.005.

Jin, Z. and Liu, Y. (2018) 'DNA methylation in human diseases', *Genes and Diseases*. Elsevier Ltd, 5(1), pp. 1–8. doi: 10.1016/j.gendis.2018.01.002.

Jofré, R. *et al.* (2006) 'Inflammatory Syndrome in Patients on Hemodialysis', *J Am Soc Nephrol*, 17, pp. 274–280. doi: 10.1681/ASN.2006080926.

Johansen, K. L. *et al.* (2007) 'Significance of frailty among dialysis patients.', *Journal of the American Society of Nephrology : JASN*, 18(11), pp. 2960–2967. doi:

10.1681/ASN.2007020221.

Johansen, K. L. *et al.* (2015) 'Characterization of Physical Activity and Sitting Time Among Patients on Hemodialysis Using a New Physical Activity Instrument', *Journal of Renal Nutrition*. doi: 10.1053/j.jrn.2014.06.012.

Johansen, K. L. (2015) 'The Frail Dialysis Population: A Growing Burden for the Dialysis Community', *Blood Purification*, 40(4), pp. 288–292. doi: 10.1159/000441575.

Johansen, K. L. *et al.* (2019) 'Frailty among patients receiving hemodialysis: Evolution of components and associations with mortality', *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, 74(3), pp. 380–386. doi: 10.1093/gerona/gly206.

Johansen, K. L., Mulligan, K. and Schambelan, M. (1999) 'Anabolic effects of nandrolone decanoate in patients receiving dialysis: A randomized controlled trial', *Journal of the American Medical Association*. doi: 10.1001/jama.281.14.1275.

Johnson, T. E. (2006) 'Recent results: Biomarkers of aging', *Experimental Gerontology*, 41(12), pp. 1243–1246. doi: 10.1016/j.exger.2006.09.006.

Jones, M. J., Goodman, S. J. and Kobor, M. S. (2015) 'DNA methylation and healthy human aging', *Aging Cell*. doi: 10.1111/acel.12349.

Jylhävä, J., Pedersen, N. L. and Hägg, S. (2017) 'Biological Age Predictors', *EBioMedicine*. The Authors, 21, pp. 29–36. doi: 10.1016/j.ebiom.2017.03.046.

Kang, S. H. *et al.* (2017) 'Effect of dialysis modality on frailty phenotype, disability, and health-related quality of life in maintenance dialysis patients', *PLoS ONE*, 12(5), pp. 1–16. doi: 10.1371/journal.pone.0176814.

Kato, S. *et al.* (2016) 'Telomere Attrition and Elongation after Chronic Dialysis Initiation in Patients with End-Stage Renal Disease', *Blood Purification*, 41(1–3), pp.

25–33. doi: 10.1159/000440971.

Khanna, A., Pradhan, A. and Curran, S. P. (2015) ‘Emerging Roles for Maf1 beyond the Regulation of RNA Polymerase III Activity’, *Journal of Molecular Biology*. doi: 10.1016/j.jmb.2015.06.022.

Kidir, V. *et al.* (2017) ‘Telomerase activity in patients with stage 2–5D chronic kidney disease’, *Nefrologia*, 37(6), pp. 592–597. doi: 10.1016/j.nefro.2017.03.025.

Kim, J. C., Kalantar-Zadeh, K. and Kopple, J. D. (2013) ‘Frailty and protein-energy wasting in elderly patients with end stage kidney disease.’, *Journal of the American Society of Nephrology : JASN*, 24(3), pp. 337–51. doi: 10.1681/ASN.2012010047.

Kim, S. and Jazwinski, S. M. (2015) ‘Quantitative measures of healthy aging and biological age’. doi: 10.12715/har.2015.4.26.Quantitative.

Kimura, M. *et al.* (2010) ‘Measurement of telomere length by the Southern blot analysis of terminal restriction fragment lengths.’, *Nature protocols*, 5(9), pp. 1596–607. doi: 10.1038/nprot.2010.124.

Kirkwood, T. B. L. (2005) ‘Understanding the odd science of aging’, *Cell*, 120(4), pp. 437–447. doi: 10.1016/j.cell.2005.01.027.

Kittiskulnam, P. *et al.* (2017) ‘Sarcopenia and its individual criteria are associated, in part, with mortality among patients on hemodialysis’, *Kidney International*. doi: 10.1016/j.kint.2017.01.024.

Kłoda, K., Domański, L. and Mierzecki, A. (2017) ‘Telomere Length Assessment for Prediction of Organ Transplantation Outcome. Future or Failure: A Review of the Literature’, *Medical Science Monitor*, 23, pp. 158–162. doi: 10.12659/MSM.899490.

Kohli, R. M. and Zhang, Y. (2013) ‘TET enzymes, TDG and the dynamics of DNA demethylation’, *Nature*. doi: 10.1038/nature12750.

Koppelstaetter, C. *et al.* (2018) ‘Effect of cyclosporine, tacrolimus and sirolimus on

cellular senescence in renal epithelial cells', *Toxicology in Vitro*, pp. 86–92. doi: 10.1016/j.tiv.2018.01.004.

Kurella Tamura, M. *et al.* (2009) 'Functional status of elderly adults before and after initiation of dialysis.', *The New England journal of medicine*, 361(16), pp. 1539–47. doi: 10.1056/NEJMoa0904655.

Kutner, N. G. (2013) 'Frailty in Dialysis-Dependent Patients With End-Stage Renal Disease', *JAMA Internal Medicine*, 173(May), pp. 351–353.

Kutner, N. G. (2014) 'Risk Factors for Frailty in a Large Prevalent Cohort of Hemodialysis Patients', *Am J Med Sci*, 6(8), pp. 277–282. doi: 10.1021/nn300902w.Release.

Kyo, S. *et al.* (1999) 'Estrogen activates telomerase', *Cancer Research*.

Laird, P. W. (2003) 'The power and the promise of DNA methylation markers', *Nature Reviews Cancer*. doi: 10.1038/nrc1045.

De Lange, T. (2004) 'T-loops and the origin of telomeres', *Nature Reviews Molecular Cell Biology*, 5(4), pp. 323–329. doi: 10.1038/nrm1359.

Langevin, S. M. *et al.* (2011) 'The influence of aging, environmental exposures and local sequence features on the variation of DNA methylation in blood', *Epigenetics*, 6(7), pp. 908–919. doi: 10.4161/epi.6.7.16431.

Lee, H. W. *et al.* (1998) 'Essential role of mouse telomerase in highly proliferative organs', *Nature*, 392(6676), pp. 569–574. doi: 10.1038/33345.

Lee, S. Y. *et al.* (2017) 'The Prevalence, Association, and Clinical Outcomes of Frailty in Maintenance Dialysis Patients', *Journal of Renal Nutrition*. Elsevier, 27(2), pp. 106–112. doi: 10.1053/j.jrn.2016.11.003.

Levine, M. E. *et al.* (2018) 'An epigenetic biomarker of aging for lifespan and healthspan', *Aging*, 10(4), pp. 573–591. doi: 10.18632/aging.101414.

Levine, M. E. and Crimmins, E. M. (2014) ‘A comparison of methods for assessing mortality risk’, *American journal of human biology : the official journal of the Human Biology Council*, 26(6), pp. 768–776. doi: 10.1002/ajhb.22595.

Libetta, C. *et al.* (2011) ‘Oxidative stress and inflammation: Implications in uremia and hemodialysis’, *Clinical Biochemistry*, pp. 1189–1198. doi: 10.1016/j.clinbiochem.2011.06.988.

Lin, J. *et al.* (2019) ‘Telomere length measurement by qPCR – Summary of critical factors and recommendations for assay design’, *Psychoneuroendocrinology*, 99, pp. 271–278. doi: 10.1016/j.psyneuen.2018.10.005.

Lin, Q. *et al.* (2016) ‘DNA methylation levels at individual age-associated CpG sites can be indicative for life expectancy’, *Aging*. doi: 10.18632/aging.100908.

Lindsey, J. *et al.* (1991) ‘In vivo loss of telomeric repeats with age in humans’, *Mutation Research DNAging*. doi: 10.1016/0921-8734(91)90032-7.

Lingner, J., Cooper, J. P. and Cech, T. R. (1995) ‘Telomerase and DNA end replication: No longer a lagging strand problem?’, *Science*. doi: 10.1126/science.7545310.

Lister, R. *et al.* (2009) ‘Human DNA methylomes at base resolution show widespread epigenomic differences’, *Nature*. doi: 10.1038/nature08514.

Lopes, A. A. *et al.* (2002) ‘Depression as a predictor of mortality and hospitalization among hemodialysis patients in the United States and Europe’, *Kidney International*, 62(1), pp. 199–207. doi: 10.1046/j.1523-1755.2002.00411.x.

Lorenzi, M. *et al.* (2018) ‘A review of telomere length in sarcopenia and frailty’, *Biogerontology*. Springer Netherlands, 19(3–4), pp. 209–221. doi: 10.1007/s10522-018-9749-5.

Lowe, D., Horvath, S. and Raj, K. (2016) ‘Epigenetic clock analyses of cellular

senescence and ageing', *Oncotarget*. doi: 10.18632/oncotarget.7383.

Lundblad, V. and Szostak, J. W. (1989) 'A mutant with a defect in telomere elongation leads to senescence in yeast', *Cell*, 57(4), pp. 633–643. doi: 10.1016/0092-8674(89)90132-3.

Luttropp, K. *et al.* (2016) 'Increased Telomere Attrition After Renal Transplantation—Impact of Antimetabolite Therapy', *Transplantation Direct*, 2(12), p. e116. doi: 10.1097/TXD.0000000000000629.

Marioni, R. E., Shah, S., McRae, A. F., Chen, B. H., *et al.* (2015) 'DNA methylation age of blood predicts all-cause mortality in later life', *Genome Biology*. doi: 10.1186/s13059-015-0584-6.

Marioni, R. E., Shah, S., McRae, A. F., Ritchie, S. J., *et al.* (2015) 'The epigenetic clock is correlated with physical and cognitive fitness in the Lothian Birth Cohort 1936', *International Journal of Epidemiology*. doi: 10.1093/ije/dyu277.

Marioni, R. E. *et al.* (2016) 'The epigenetic clock and telomere length are independently associated with chronological age and mortality', *International Journal of Epidemiology*, 45(2), pp. 424–432. doi: 10.1093/ije/dyw041.

Martin-Ruiz, C. M. *et al.* (2005) 'Telomere length in white blood cells is not associated with morbidity or mortality in the oldest old: A population-based study', *Aging Cell*, 4(6), pp. 287–290. doi: 10.1111/j.1474-9726.2005.00171.x.

Martino, D. *et al.* (2013) 'Longitudinal, genome-scale analysis of DNA methylation in twins from birth to 18 months of age reveals rapid epigenetic change in early life and pair-specific effects of discordance', *Genome Biology*. doi: 10.1186/gb-2013-14-5-r42.

Marzetti, E. *et al.* (2014) 'Shorter telomeres in peripheral blood mononuclear cells from older persons with sarcopenia : results from an exploratory study', 6(August),

pp. 1–8. doi: 10.3389/fnagi.2014.00233.

Masser, D. R. *et al.* (2017) 'Sexually divergent DNA methylation patterns with hippocampal aging', *Aging Cell*. doi: 10.1111/acel.12681.

Matosin, N., Cruceanu, C. and Binder, E. B. (2017) 'Preclinical and Clinical Evidence of DNA Methylation Changes in Response to Trauma and Chronic Stress', *Chronic Stress*. doi: 10.1177/2470547017710764.

McAdams-Demarco, M. A. *et al.* (2013) 'Frailty as a novel predictor of mortality and hospitalization in individuals of all ages undergoing hemodialysis', *Journal of the American Geriatrics Society*, 61(6), pp. 896–901. doi: 10.1111/jgs.12266.

McClintock, B. (1939) 'The Behavior in Successive Nuclear Divisions of a Chromosome Broken at Meiosis', *Proceedings of the National Academy of Sciences*, 25(8), pp. 405–416. doi: 10.1073/pnas.25.8.405.

McCurdy, R. D., McGrath, J. J. and Mackay-Sim, A. (2008) 'Validation of the comparative quantification method of real-time PCR analysis and a cautionary tale of housekeeping gene selection', *Gene Therapy and Molecular Biology*, 12(1), pp. 15–24.

McEwen, B. S. and Stellar, E. (1993) 'Stress and the Individual: Mechanisms Leading to Disease', *Archives of Internal Medicine*. doi: 10.1001/archinte.1993.00410180039004.

Medvedev, Z. A. (1990) 'An Attempt At A Rational Classification Of Theories Of Ageing', *Biological Reviews*. Blackwell Publishing Ltd, 65(3), pp. 375–398. doi: 10.1111/j.1469-185X.1990.tb01428.x.

Meijers, R. W. J. *et al.* (2012) 'Uremia causes premature ageing of the T cell compartment in end-stage renal disease patients', *Immunity and Ageing*. Immunity & Ageing, 9(1), p. 1. doi: 10.1186/1742-4933-9-19.

Michel, J. P., Cruz-Jentoft, A. J. and Cederholm, T. (2015) 'Frailty, Exercise and Nutrition', *Clinics in Geriatric Medicine*. doi: 10.1016/j.cger.2015.04.006.

Mitchell, J. R. and Collins, K. (2000) 'Human telomerase activation requires two independent interactions between telomerase RNA and telomerase reverse transcriptase', *Molecular Cell*, 6(2), pp. 361–371. doi: 10.1016/S1097-2765(00)00036-8.

Mitnitski, A. B. *et al.* (2002) 'Frailty, fitness and late-life mortality in relation to chronological and biological age', *BMC Geriatrics*, 2(1), p. 1. doi: 10.1186/1471-2318-2-1.

Müezzinler, A., Zaineddin, A. K. and Brenner, H. (2013) 'A systematic review of leukocyte telomere length and age in adults', *Ageing Research Reviews*. Elsevier B.V., 12(2), pp. 509–519. doi: 10.1016/j.arr.2013.01.003.

Muller, H. (1938) 'The remaking of chromosomes', *Collecting Net*, 13, pp. 181–198. Available at: http://ci.nii.ac.jp/naid/10006827930/en/ (Accessed: 22 November 2017).

Murillo-Ortiz, B. *et al.* (2016) 'Impact of Oxidative Stress in Premature Aging and Iron Overload in Hemodialysis Patients', *Oxidative Medicine and Cellular Longevity*, 2016. doi: 10.1155/2016/1578235.

Ng, L. J. *et al.* (2009) 'Telomerase activity is associated with an increase in DNA methylation at the proximal subtelomere and a reduction in telomeric transcription', *Nucleic Acids Research*. doi: 10.1093/nar/gkn1030.

Ní Mhaoláin, A. M. *et al.* (2012) 'Frailty, depression, and anxiety in later life', *International Psychogeriatrics*. doi: 10.1017/S1041610211002110.

Nitta, K., Hanafusa, N. and Tsuchiya, K. (2017) 'Frailty and mortality among dialysis patients', *Renal Replacement Therapy*. Renal Replacement Therapy, 3(1), p. 41. doi: 10.1186/s41100-017-0122-y.

Oikawa, S. and Kawanishi, S. (1999) ‘Site-specific DNA damage at GGG sequence by oxidative stress may accelerate telomere shortening’, *FEBS Letters*, 453(3), pp. 365–368. doi: 10.1016/S0014-5793(99)00748-6.

Oikawa, S., Tada-Oikawa, S. and Kawanishi, S. (2001) ‘Site-specific DNA damage at the GGG sequence by UVA involves acceleration of telomere shortening’, *Biochemistry*, 40(15), pp. 4763–4768. doi: 10.1021/bi002721g.

Okano, M. *et al.* (1999) ‘DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development’, *Cell*. doi: 10.1016/S0092-8674(00)81656-6.

Okuda, K. *et al.* (2002) ‘Telomere length in the newborn’, *Pediatric Research*. doi: 10.1203/00006450-200209000-00012.

Olovnikov, A. M. (1973) ‘A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon’, *Journal of Theoretical Biology*. Academic Press, 41(1), pp. 181–190. doi: 10.1016/0022-5193(73)90198-7.

Olovnikov, A. M. (1996) ‘Telomeres, telomerase, and aging: Origin of the theory’, *Experimental Gerontology*, 31(4), pp. 443–448. doi: 10.1016/0531-5565(96)00005-8.

Painter, P. and Kuskowski, M. (2013) ‘A closer look at frailty in ESRD: Getting the measure right’, *Hemodialysis International*. doi: 10.1111/j.1542-4758.2012.00719.x.

Palm, W. and de Lange, T. (2008) ‘How Shelterin Protects Mammalian Telomeres’, *Annual Review of Genetics*, 42(1), pp. 301–334. doi: 10.1146/annurev.genet.41.110306.130350.

Palmer, S. *et al.* (2013) ‘Prevalence of depression in chronic kidney disease: Systematic review and meta-analysis of observational studies’, *Kidney International*. doi: 10.1038/ki.2013.77.

Park, S. L. *et al.* (2018) 'Association of internal smoking dose with blood DNA methylation in three racial/ethnic populations', *Clinical Epigenetics*. doi: 10.1186/s13148-018-0543-7.

Passos, F. *et al.* (2007) 'Mitochondrial Dysfunction Accounts for the Stochastic Heterogeneity in Telomere-Dependent Senescence', *Plos Biology*, 5(5). doi: 10.1371/journal.pbio.0050110.

Pertosa, G. *et al.* (2000) 'Clinical relevance of cytokine production in hemodialysis.', *Kidney international. Supplement*, 76, pp. S104-11. doi: 10.1046/j.1523-1755.2000.07613.x.

Petersen, S., Saretzki, G. and Von Zglinicki, T. (1998) 'Preferential accumulation of single-stranded regions in telomeres of human fibroblasts', *Experimental Cell Research*, 239(1), pp. 152–160. doi: 10.1006/excr.1997.3893.

Petkovich, D. A. *et al.* (2017) 'Using DNA Methylation Profiling to Evaluate Biological Age and Longevity Interventions', *Cell Metabolism*. Elsevier Inc., 25(4), p. 954–960.e6. doi: 10.1016/j.cmet.2017.03.016.

Pinto, A. P. *et al.* (2015) 'Impact of hemodialysis session on handgrip strength', *Jornal brasileiro de nefrologia : 'orgao oficial de Sociedades Brasileira e Latino-Americana de Nefrologia*. doi: 10.5935/0101-2800.20150072.

Pradhananga, S. *et al.* (2019) 'Ethnic differences in the prevalence of frailty in the United Kingdom assessed using the electronic Frailty Index', *Aging Medicine*, (August), pp. 1–6. doi: 10.1002/agm2.12083.

Rakyan, V. K. *et al.* (2010) 'Human aging-associated DNA hypermethylation occurs preferentially at bivalent chromatin domains', *Genome Research*. doi: 10.1101/gr.103101.109.

Ramírez, R. *et al.* (2005) 'Stress-induced premature senescence in mononuclear cells

from patients on long-term hemodialysis', *American Journal of Kidney Diseases*, 45(2), pp. 353–359. doi: 10.1053/j.ajkd.2004.10.022.

Roderick, P. A. U. L. J. R. *et al.* (2007) 'Detecting chronic kidney disease in older people ; what are the implications ?', (December 2007), pp. 179–186. doi: 10.1093/ageing/afm180.

S., C. *et al.* (2013) 'Endogenous testosterone, muscle strength, and fat-free mass in men with chronic kidney disease', *Journal of Renal Nutrition*.

Sampson, M. J. *et al.* (2006) 'Monocyte telomere shortening and oxidative DNA damage in type 2 diabetes', *Diabetes Care*. doi: 10.2337/diacare.29.02.06.dc05-1715.

Sandin, S. and Rhodes, D. (2014) 'Telomerase structure', *Current Opinion in Structural Biology*. Elsevier Ltd, 25, pp. 104–110. doi: 10.1016/j.sbi.2014.02.003.

Saum, K. U. *et al.* (2014) 'Frailty and telomere length: Cross-sectional analysis in 3537 older adults from the ESTHER cohort', *Experimental Gerontology*. Elsevier Inc., 58, pp. 250–255. doi: 10.1016/j.exger.2014.08.009.

Sela, S. (2005) 'Primed Peripheral Polymorphonuclear Leukocyte: A Culprit Underlying Chronic Low-Grade Inflammation and Systemic Oxidative Stress in Chronic Kidney Disease', *Journal of the American Society of Nephrology*, 16(8), pp. 2431–2438. doi: 10.1681/ASN.2004110929.

Sharman, A. and Zhumadilov, Z. (2011) *The Scientific Basis for Healthy Aging and Antiaging Processes*.

Shlipak, M. G. *et al.* (2004) 'The presence of frailty in elderly persons with chronic renal insufficiency', *American Journal of Kidney Diseases*. National Kidney Foundation, Inc., 43(5), pp. 861–867. doi: 10.1053/j.ajkd.2003.12.049.

Sillanpaä, E. *et al.* (2018) 'Biological clocks and physical functioning in monozygotic female twins', *BMC Geriatrics*. doi: 10.1186/s12877-018-0775-6.

Simpkin, A. J., Suderman, M. and Howe, L. D. (2017) 'Epigenetic clocks for gestational age: Statistical and study design considerations', *Clinical Epigenetics*. doi: 10.1186/s13148-017-0402-y.

Spiegel, J., Adhikari, S. and Balasubramanian, S. (2019) 'The Structure and Function of DNA G-Quadruplexes', *Trends in Chemistry*. The Author(s), xx(xx), pp. 1–14. doi: 10.1016/j.trechm.2019.07.002.

Stefanidis, I. *et al.* (2015) 'Telomere Length in Peripheral Blood Mononuclear Cells of Patients on Chronic Hemodialysis Is Related With Telomerase Activity and Treatment Duration', *Artificial Organs*, 39(9), pp. 756–764. doi: 10.1111/aor.12453.

Stenvinkel, P. *et al.* (2007) 'Impact of inflammation on epigenetic DNA methylation - A novel risk factor for cardiovascular disease?', *Journal of Internal Medicine*, 261(5), pp. 488–499. doi: 10.1111/j.1365-2796.2007.01777.x.

Stenvinkel, P. *et al.* (2008) 'Emerging biomarkers for evaluating cardiovascular risk in the chronic kidney disease patient: How do new pieces fit into the uremic puzzle?', *Clinical Journal of the American Society of Nephrology*, 3(2), pp. 505–521. doi: 10.2215/CJN.03670807.

Stenvinkel, P. and Ekström, T. J. (2008) 'Epigenetics - A helpful tool to better understand processes in clinical nephrology?', *Nephrology Dialysis Transplantation*, 23(5), pp. 1493–1496. doi: 10.1093/ndt/gfn056.

Stenvinkel, P. and Larsson, T. E. (2013) 'Chronic kidney disease: a clinical model of premature aging.', *American journal of kidney diseases : the official journal of the National Kidney Foundation*, 62(2), pp. 339–51. doi: 10.1053/j.ajkd.2012.11.051.

Steves, C. J., Spector, T. D. and Jackson, S. H. D. (2012) 'Ageing, genes, environment and epigenetics: What twin studies tell us now, and in the future', *Age and Ageing*, 41(5), pp. 581–586. doi: 10.1093/ageing/afs097.

Sumida, K. *et al.* (2016) 'Different impact of hemodialysis vintage on cause-specific mortality in long-term hemodialysis patients', *Nephrology Dialysis Transplantation*, 31(2), pp. 298–305. doi: 10.1093/ndt/gfv402.

Sun, J. *et al.* (2016) 'Biomarkers of cardiovascular disease and mortality risk in patients with advanced CKD', *Clinical Journal of the American Society of Nephrology*, 11(7), pp. 1163–1172. doi: 10.2215/CJN.10441015.

Szanton, S. L. *et al.* (2010) 'Socioeconomic status is associated with frailty: The Women's Health and Aging Studies', *Journal of Epidemiology and Community Health*. doi: 10.1136/jech.2008.078428.

Talens, R. P. *et al.* (2012) 'Epigenetic variation during the adult lifespan: Cross-sectional and longitudinal data on monozygotic twin pairs', *Aging Cell*. doi: 10.1111/j.1474-9726.2012.00835.x.

Tan, Q. *et al.* (2016) 'Epigenetic drift in the aging genome: a ten-year follow-up in an elderly twin cohort.', *International journal of epidemiology*. doi: 10.1093/ije/dyw132.

Tarng, D. C. *et al.* (2000) '8-hydroxy-2'-deoxyguanosine of leukocyte DNA as a marker of oxidative stress in chronic hemodialysis patients.', *American journal of kidney diseases : the official journal of the National Kidney Foundation*, 36(5), pp. 934–44. doi: 10.1053/ajkd.2000.19086.

Tran, P. T., Meeker, A. K. and Platz, E. A. (2018) 'Association between statin drug use and peripheral blood leukocyte telomere length in the National Health and Nutrition Examination Survey 1999–2002: a cross-sectional study', *Annals of Epidemiology*. doi: 10.1016/j.annepidem.2018.04.010.

Tsirpanlis, G. *et al.* (2006) 'Telomerase activity is decreased in peripheral blood mononuclear cells of hemodialysis patients', *American Journal of Nephrology*, 26(1), pp. 91–96. doi: 10.1159/000092031.

Vanyushin, B. F. *et al.* (1973) 'The 5-Methylcytosine in DNA of Rats', *Gerontology*. doi: 10.1159/000211967.

Vasan, R. S. *et al.* (2008) 'Association of leukocyte telomere length with circulating biomarkers of the renin-angiotensin-aldosterone system: The Framingham heart study', *Circulation*, 117(9), pp. 1138–1144. doi: 10.1161/CIRCULATIONAHA.107.731794.

Vaziri, H. *et al.* (1994) 'Evidence for a mitotic clock in human hematopoietic stem cells: loss of telomeric DNA with age.', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.91.21.9857.

Vera, E. *et al.* (2012) 'The Rate of Increase of Short Telomeres Predicts Longevity in Mammals', *Cell Reports*. doi: 10.1016/j.celrep.2012.08.023.

Vilkaitis, G. *et al.* (2005) 'Processive methylation of hemimethylated CpG sites by mouse Dnmt1 DNA methyltransferase', *Journal of Biological Chemistry*. doi: 10.1074/jbc.M411126200.

Vogt, B. P. *et al.* (2016) 'Handgrip strength is an independent predictor of all-cause mortality in maintenance dialysis patients', *Clinical Nutrition*. doi: 10.1016/j.clnu.2016.03.020.

Vogt, M. and Haggblom, C. (1998) 'Independent induction of senescence by p16INK4a and p21CIP1 in spontaneously immortalized human fibroblasts.', *Cell growth & …*.

De Vries, N. *et al.* (2018) 'A15812 Angiotensin Converting Enzyme Inhibitors Epigenetically Atenuate Telomere Shortening', *Journal of Hypertension*. doi: 10.1097/01.hjh.0000548337.09432.ae.

Wang, L. J. *et al.* (2012) 'The relationship between psychological factors, inflammation, and nutrition in patients with chronic renal failure undergoing

hemodialysis', *International Journal of Psychiatry in Medicine*. doi: 10.2190/PM.44.2.b.

Wang, T. *et al.* (2017) 'Epigenetic aging signatures in mice livers are slowed by dwarfism, calorie restriction and rapamycin treatment', *Genome Biology*. doi: 10.1186/s13059-017-1186-2.

Watson, J. D. and Crick, F. H. C. (1969) 'Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid (Reprinted from Nature, April 25, 1953)', *Nature*, 224(5218), pp. 470–471. doi: 10.1038/224470a0.

Whittemore, K. *et al.* (2019) 'Telomere shortening rate predicts species life span', *Proceedings of the National Academy of Sciences*, 0, p. 201902452. doi: 10.1073/pnas.1902452116.

Wilson, V. L. *et al.* (1987) 'Genomic 5-methyldeoxycytidine decreases with age.', *Journal of Biological Chemistry*.

Wilson, V. L. and Jones, P. A. (1983) 'DNA methylation decreases in aging but not in immortal cells', *Science*. doi: 10.1126/science.6844925.

Wong, J. Y. Y. *et al.* (2014) 'The Relationship between Inflammatory Biomarkers and Telomere Length in an Occupational Prospective Cohort Study', 9(1). doi: 10.1371/journal.pone.0087348.

Woo, J. *et al.* (2008) 'Telomeres and frailty', 129, pp. 642–648. doi: 10.1016/j.mad.2008.08.003.

Woods, N. F. *et al.* (2005) 'Frailty: Emergence and consequences in women aged 65 and older in the Women's Health Initiative observational study', *Journal of the American Geriatrics Society*, 53(8), pp. 1321–1330. doi: 10.1111/j.1532-5415.2005.53405.x.

Wright, W. E. *et al.* (1997) 'Normal human chromosomes have long G-rich telomeric

overhangs at one end', *Genes and Development*, 11(21), pp. 2801–2809. doi: 10.1101/gad.11.21.2801.

Wyatt, H. D. M., West, S. C. and Beattie, T. L. (2010) 'InTERTpreting telomerase structure and function', *Nucleic Acids Research*, 38(17), pp. 5609–5622. doi: 10.1093/nar/gkq370.

Xiao, F. H., Wang, H. T. and Kong, Q. P. (2019) 'Dynamic DNA methylation during aging: A "prophet" of age-related outcomes', *Frontiers in Genetics*. doi: 10.3389/fgene.2019.00107.

Xue, Q. L. *et al.* (2008) 'Initial manifestations of frailty criteria and the development of frailty phenotype in the women's health and aging study II', *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, 63(9), pp. 984–990. doi: 10.1093/gerona/63.9.984.

Yang, H. and Fogo, A. B. (2010) 'Cell Senescence in the Aging Kidney', *Journal of the American Society of Nephrology*, 21(9), pp. 1436–1439. doi: 10.1681/ASN.2010020205.

Yoda, M. *et al.* (2012) 'Poor muscle quality as a predictor of high mortality independent of diabetes in hemodialysis patients', *Biomedicine and Pharmacotherapy*. doi: 10.1016/j.biopha.2011.11.001.

Yoo, J. *et al.* (2017) 'Biological age as a useful index to predict seventeen-year survival and mortality in Koreans', *BMC Geriatrics*. BMC Geriatrics, 17(1), p. 7. doi: 10.1186/s12877-016-0407-y.

Yu, R. *et al.* (2015) 'Telomere length is not associated with frailty in older Chinese elderly : Cross-sectional and longitudinal analysis', *Mechanisms of Ageing and Development*. Elsevier Ireland Ltd, 152, pp. 74–79. doi: 10.1016/j.mad.2015.10.002.

Yuan, X. *et al.* (2018) 'Longitudinal changes in leukocyte telomere length and

mortality in elderly Swedish men', *Aging*, 10(10), pp. 3005–3016. doi: 10.18632/aging.101611.

Von Zglinicki, T. (2002) 'Oxidative stress shortens telomeres', *Trends in Biochemical Sciences*, 27(7), pp. 339–344. doi: 10.1016/S0968-0004(02)02110-2.

Zglinicki, T. Von, Martin-ruiz, C. M. and Saretzki, G. (2005) 'Telomeres , cell senescence and human ageing', *Signal Transduction*. doi: 10.1002/sita.200400049.

Von Zglinicki, T., Pilger, R. and Sitte, N. (2000) 'Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts', *Free Radical Biology and Medicine*, 28(1), pp. 64–74. doi: 10.1016/S0891-5849(99)00207-5.

Zhang, C. (2014) 'The correlation between iron homeostasis and telomere maintenance', *Frontiers in Biology*. doi: 10.1007/s11515-014-1327-x.

Zhang, W. G. *et al.* (2018) 'Peripheral blood leukocyte telomere length is associated with age but not renal function: A cross-sectional follow-up study', *Journal of Nutrition, Health and Aging*. doi: 10.1007/s12603-017-0905-4.

Zhu, X. *et al.* (2016) 'The association between telomere length and cancer risk in population studies', *Scientific Reports*. Nature Publishing Group, 6, pp. 1–10. doi: 10.1038/srep22243.

Appendices

Appendix A: Study Protocol Version 4.0

Appendix B: Full set of project data

Appendix C: Patient information sheet version 2

Appendix D: Consent form version 2

Appendix E: GP letter version 1

Appendix F: Frailty Measurement Part 1 and Overall Progress

Appendix G: Frailty Measurement Part 2: Minnesota Leisure-Time Physical Activity Questionnaire

Appendix H: CpG ID's of Probes Used in Measuring DNA Methylation Status